31 Published Papers on the Amino Acid Composition and Titration Curve of Collagen

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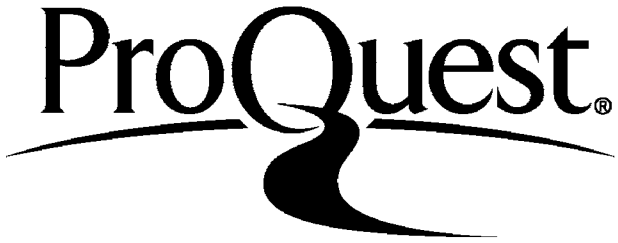

Titles and Dates of Published Papers.

Group I. The Composition and Properties of the Skin Proteins.

1. The Amino-acid Composition and Titration Curve of Collagen.

   J.H. Bowes and R.H. Kenten, Biochem. J. 1948, 43, 358.

2. The Effect of Alkalis on Collagen.

   J.H. Bowes and R.H. Kenten, Biochem.J. 1948, 43, 365.

3. The Effect of Deamination and Esterification on the Reactivity of Collagen.

   J.H. Bowes and R.H. Kenten, Biochem.J. 1949, 44, 142.

4. The Effect of Modification of the Reactive Groups of Collagen on the Fixation of Tanning Agents.

   J.H. Bowes and R.H. Kenten, J.Soc. Leather Trades' Chem. 1949, 38, 368.

5. Some Observations on the Amino-acid Distribution of Collagen, Elastin and Reticular Tissue from Different Sources.

   J.H. Bowes and R.H. Kenten, Biochem. J. 1949, 45, 281.

*6. The Composition and Reactivity of Collagen.

   J.H. Bowes - paper given to the 1st International Congress of Biochemistry, Cambridge, 1949.

7. Uptake of Water by Collagen in Solutions of Alkalis and Strong and Weak Organic Bases.

   J.H. Bowes and R.H. Kenten, Nature, 1947, 160, 827.

8. The Swelling of Collagen in Alkaline Solutions.

1. Swelling in Sodium Hydroxide Solutions.

J.H. Bowes and R.H. Kenten, Biochem. J. 1950, 46, 1.

9. II. Swelling in Solutions of Univalent Bases.

J.H. Bowes and R.H. Kenten, Biochem. J. 1950, 46, 524

10. III. Swelling in Solutions of Bivalent Bases.

J.H. Bowes, Biochem.J. 1950, 46, 530.

11. Recent Studies of the Chemistry of the Liming Process. A review.

J.H. Bowes, J. Soc. Leather Trades' Chem. 1951, 33, 176.

12. Free Amino Groups of Collagen.

J.H. Bowes and J.A. Moss, Nature 1951, 168, 514.

*13. Application of the End Group Technique to the Study of Collagen.

J.H. Bowes and J.A. Moss - paper given to the 2nd International Congress of Biochemistry, Paris, 1952.

14. The Reaction of Fluorodinitrobenzene with the α and ε -amino Groups of Collagen.

J.H. Bowes and J.A. Moss, Biochem. J. 1953, 55, 735.

15. Some Differences in the Composition of Collagen and Extracted Collagen and their Relation to Fibre Formation and Dispersion.

J.H. Bowes, R.G. Elliott and J.A. Moss, Nature and Structure of Collagen, Butterworth, 1953, p.199.

16. The Composition of Collagen and Acid-Soluble Collagen of Bovine Skin.

J.H. Bowes, R.G. Elliott and J.A. Moss, Biochem. J. 1955, 61, 143.

17. The Composition of Some Protein Fractions Isolated from Bovine Skin.

Contribution to Symposium on Connective Tissue organised by Council for International Organizations of Medical Sciences, London, July, 1956.

J.H. Bowes, R.G. Elliott and J.A.Moss - in the press.

18. Collagen and the More Soluble Constituents of Skin - A review.

J.H. Bowes, R.G. Elliott and J.A. Moss J. Soc. Leather Trades' Chem. 1957, 41, 249.

19. The Composition of Epidermis.

J.H. Bowes and R.G. Elliott, J. Soc. Leather Trades' Chem. 1957, 41, 87.

20. The Extraction of Soluble Protein from Skin by Alkaline Solutions.

J.H. Bowes, R.G. Elliott and J.A. Moss - paper given at the British Gelatine and Glue Research Association Conference on Recent Advances in Gelatine and Glue Researc Cambridge - July, 1957. - in the press.

Group II. The Preparation of Skins for Tanning, Tanning and the Physical Properties of Leather.

1. Formaldehyde Tanning.

I. The Combination of Formaldehyde with Collagen, Keratin and Silk Fibroin.

2. II. The Combination of Collagen and Keratin with Formaldehyde in the Presence of Calcium Hydroxide.

3. III. The Combination of Formaldehyde with Deaminated Collagen and Keratin.

4. IV. The Influence of Time on the Combination of Formaldehyde with Collagen and Keratin.

J.H. Bowes and W.B. Pleass, J. Soc. Leather Trades' Chem. 1939, 23, 365, 451, 453 and 499.

5. Experiments on Treatment of Hides with Solutions of Washing Soda for Surface Disinfection after Contact with Foot-and-Mouth Disease.

J.H. Bowes, R. Inkster and W.B. Ple
J. Soc. Leather Trades' Chem. 1940, 24, 105.

6. The Use of Amines in the Fellmongering of Sheepskins and the Liming of Goatskins.

J.H. Bowes and W.B. Pleass. J. Soc
Leather Trades' Chemists 1942, 26, 140.

7. "Run" in Gloving Leather.

II. The Relationship between "Run" and Certain Physical Properties.

III. The Effect of Straining and Crushing on the Flexural Rigidity.

J.H. Bowes, J. Soc. Leather Trade
Chem. 1942, 26, 18 and 204.

8. The Effect of Storage in the Salted and in the Pickled Condition, on the Fibre Structure and Grease Content of Sheepskins and Goatskins.

M.P. Balfe, J.H. Bowes, R.F. Innes and W.B. Pleass. J. Soc. Leather Trades' Chem. 1940, 24, 329.

9. The Use of Salts in the Finishing of Sole Bends.

I. The Precipitation of Tannins by Salts.

10. 2. The Effect of Cane Sugar and Other Organic Compounds on the Prevention of Salt Spue.

J.H. Bowes, J. Soc. Leather Trades' Chem. 1948, 32, 224, and 275.

11. 3. The Effect of Magnesium Sulphate, Cane Sugar and Glucose on the Drying of Leather and on the Water Content of Leathers at Different Humidities.

J.H. Bowes and G.O. Morse, J. Soc. Leather Trades' Chem. 1948, 32, 343.

12. 4. The Effect of Immersion in Solutions of Magnesium Sulphate, Cane Sugar and Glucose on Some Physical Properties of the Finished Leather.

J.H. Bowes, F.H. Quinn and C.L. Ward. J. Soc. Leather Trades' Chem. 1948, 32, 377.

13. A Note on the Calculation of Hide Substance from Nitrogen Determination.

J.H. Bowes and R.H. Kenten. J. Soc. Leather Trades' Chem. 1948, 32, 308.

14. The Yellowing of Pickled Sheepskins.

J.H. Bowes and G.O. Morse, J. Soc. Leather Trades' Chem. 1951, 35, 133.

Reviews

16. Reports of the Progress of Applied Chemistry - Leather.

1942, 27, 357

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*17. Progress in Leather Science 1920-45

British Leather Manufacturers' Research Association, London, 1948.

Chapter on I. Liming, p. 158-193.

2. The Reaction of Formaldehyde with Amino Acids and Proteins with special reference to Formaldehyde Tanning. p. 501-518.

3. The Chemistry of Mineral Tanning, p.519-548

* No reprints available

† Three reprints only available

Group I. The Composition and Properties of the Skin Proteins.

The work reported in these papers was initiated and supervised by me. Part of the work reported in papers 12-18 forms the basis of the thesis being submitted to London University by Mr.J.A. Moss for the Degree of Doctor of Philosophy (Biochemistry).

Group II. The Preparation of Skins for Tanning, Tanning and the Physical Properties of Leather.

Papers 1 to 8 - I was the junior author and responsible for the carrying out of the experimental work. I was directly responsible for the initiation and direction of the work reported in the remainder of the papers.

D. Sc (Edinburgh) 1958.

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The Amino-acid Composition and Titration Curve of Collagen

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The British Leather Manufacturers' Research Association, London, S.E. 1

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The amino-acid composition of gelatin has been extensively studied, but until recently little attention has been paid to that of its precursor collagen, the natural protein of skin. Some of the analyses of collagen which have been reported were carried out by methods which are now considered unsatisfactory, and in no case have most of the major component amino-acids been determined in one sample of the protein. Further, most of the analyses have been made on collagens which had been given an alkaline or enzyme treatment, or both, to remove the keratins, elastic fibres and reticular tissue which occur in close association with it, and there is a risk that such treatments will cause some modification of the collagen.

For the present study, a sample of collagen has been prepared with the minimum of chemical treatment, since it was considered that the presence of small amounts of elastic fibres and reticular tissue would lead to less error than the treatments necessary to bring about their removal. Also, it seems doubtful whether it is possible to remove these proteins completely by any of the methods which have been suggested. On this preparation Chibnall and his collaborators (Chibnall, 1946; Macpherson, 1946; Rees, 1946; Tristram, 1946) have determined

the basic and hydroxyamino acids, and the following neutral amino-acids: alanine, leucine, isoleucine, valine, phenylalanine and proline; the present authors have determined total N, amino N, amide N, glutamic and aspartic acids, and methionine.

The acid and basic amino-acid contents may also be deduced from the titration curve of a protein. This procedure has an advantage over analysis for the individual amino-acids in that it gives information regarding the reactive groups, including terminal groups, in the intact protein, and comparison of the results with those obtained by direct analysis can give information regarding the way in which the amino-acids are linked in the protein molecule (Chibnall, 1942; Cannan, Kibrick & Palmer, 1941, 1942).

It must be observed, however, that the titration curve of a fibrous protein only gives an exact indication of the titratable groups when it is determined in the presence of salts (Speakman & Hirst, 1933; Jordan Lloyd & Bidder, 1934; Steinhardt & Harris, 1940). This effect of salt is due in the first place to its influence on the Donnan equilibrium. The effective pH value for the equilibrium

$$R\mathrm{COO}^- + \mathrm{H}^+ \rightleftharpoons R\mathrm{COOH}$$

is the pH value in the protein phase; in the absence of salts this pH value is higher than that in the external solution, except at very low pH values, and so the binding of hydrogen ions is less than that corresponding to the external pH value. It has also been suggested (Gilbert & Rideal, 1944) that stoicheiometric binding of hydrogen ions by fibrous proteins only occurs when some anions are also bound in order to lessen the large potential which would otherwise exist between fibre and solution. The addition of salts, by increasing the concentration of anions, will facilitate this binding, and thus favour stoicheiometric binding of hydrogen ions (see also Steinhardt & Harris, 1940). Similar arguments apply to titration with alkali. The concentration of salt necessary for stoicheiometric binding depends on the protein; with collagen it has been shown to be 0·1 M or greater (Retterova, private communication).

In the present investigation the titration curve has been determined in the absence of salts, and also in the presence of 0·5 M-sodium chloride.

METHODS

Preparation of collagen. The back area of a freshly flayed ox hide was cut into pieces (*c.* 18 × 12 in.), which were washed in a revolving glass drum, first with water and then with 10% NaCl for 30 min. The pieces were left stationary overnight in a fresh NaCl solution, drummed for 30 min. in this solution, washed in several changes of distilled water, and dehydrated with acetone. The grain layer (containing the hair roots, and the greater part of the muscle and elastic fibres) and a thin layer from the flesh side were split off, and the remaining material cut into 1 cm. cubes. The cubes were degreased with three changes of light petroleum (b.p. 40-60°) at room temperature for 6 days, washed with successive changes of distilled water, and dehydrated with acetone. The purified collagen contained ash 0·03, moisture 23·0 and grease <0·1%.

Analyses. Total N was determined by the method of Chibnall, Rees & Williams (1943), and amide N by the method of Bailey (1937) and Lugg (1938). The amino N was determined by the Van Slyke manometric procedure, using an auxiliary reaction vessel as described by Doherty & Ogg (1943). To ensure thorough wetting of the sample, the finely shaved collagen (0·15–0·20 g.) was placed in the extraction vessel with 2 ml. of water, the vessel evacuated while shaking, and left under vacuum overnight. The vessel was re-evacuated immediately before the determination.

Dicarboxylic acids were determined by the method of Consden, Gordon & Martin (1948), and methionine by the method of Baernstein (1932).

Titration curves. Collagen (0·75 g.) was placed in 75 ml. of solutions containing measured volumes of HCl and NaOH in resistance glass vessels at 20°. For the titration curve in the presence of salt, sufficient NaCl was added to give a final Cl^- concentration of 0·5 M in the acid solutions, and a final Na^+ concentration of 0·5 M in the alkaline solutions. After 3 days, in which time equilibrium was known to have been reached, samples were withdrawn for pH determinations, and for titration with acid or alkali using bromocresol purple as indicator. When the final solution was too dilute for titration (between pH 3 and 11) the concentration of acid or alkali was obtained from the pH by the use of blank curves for water and for 0·5 M-NaCl; this avoided the necessity of choosing suitable activity coefficients for the two systems. In the more concentrated solutions, the amount of acid or base bound by the collagen is small compared with that remaining in solution, and larger samples of collagen (2 g. in 100 ml.) were used.

pH values were determined at 20° using a glass electrode assembly mounted in a thermostat (Coates, 1945). pH values above 9·0 were determined with a glass electrode of low sodium error (Dole, 1941).

In determining the amounts of alkali bound, precautions were taken to avoid contamination with CO_2. The collagen was placed in the vessel shown in Fig. 1*a*, and the air displaced with CO_2-free air passed in by the tube *B*. The solution of alkali was then added, the tube *A* closed with a small rubber bung, and *B* by a rubber cap. Samples for titration were taken by inserting a pipette at *A*, carrying a similar rubber bung, and forcing the solution into this pipette by passing CO_2-free air into the vessel at *B*. The titration was carried out in a vessel through which a stream of CO_2-free air was continuously blown. In the determination of pH, the solution was forced up into the electrode vessel in a similar way (see Fig. 1*b*), or, in the case of the electrode system mounted in the thermostat, a sample was transferred by means of a pipette.

The soluble N in some of the solutions below pH 2·0 and above pH 12 was determined by micro-Kjeldahl (Markham, 1942) to give an indication of the extent of decomposition, but no attempt was made to apply a correction, owing to lack of information concerning the origin of this N.

Estimate of errors involved in the determination. Errors may arise from the following causes: (1) Difference in the pH value between the solution inside the collagen and the

external solution. (2) Introduction of water with the collagen, thus increasing the total volume of solution. (3) Binding of water by the collagen, thus decreasing the amount of water available for solution of the acid or alkali. (4) Decomposition of the collagen, or liberation of groups not originally present in the native collagen.

Table 1. *Soluble nitrogen derived from collagen after 3 days' contact with acid or alkali*

In the absence of salt		In the presence of 0·5 M-NaCl	
pH	mg. N/g. collagen	pH	mg. N/g. collagen
1·58	0·14	1·64	0·95
1·68	0·12	1·73	0·85
1·80	0·15	1·85	0·77
—	—	2·00	0·69
—	—	2·10	0·70
12·38	1·54	12·46	1·04
12·59	1·72	12·59	1·28
—	—	12·66	1·21
12·78	1·95	12·71	1·08
12·92	2·22	12·86	1·44
13·16	3·86	13·12	3·08

0·03 mmol./g., and at pH 2·0, 0·003 mmol./g. The fact that the curves show well-defined maxima in the acid and alkaline ranges confirms that the net error must be small.

The soluble N present in the solutions (see Table 1) indicates that decomposition of the collagen was small even in the most acid and alkaline solutions. The chief source of this N is probably NH_3 from hydrolysis of amide groups. Under the conditions of the experiment the greater part of this NH_3 will be titrated with acid, thus compensating for the alkali bound by the freed carboxyl groups, and hence such decomposition will not affect the calculation of the amount of alkali bound by the original collagen.

RESULTS

Amino-acid composition

The collagen, prepared as described, may be considered to be a reasonably pure sample. Of the other proteins and tissues present in the fresh skin, albumins and globulins will be removed by treatment in sodium chloride solution (Kritzinger, 1948), and the greater part of the keratins, elastic fibres,

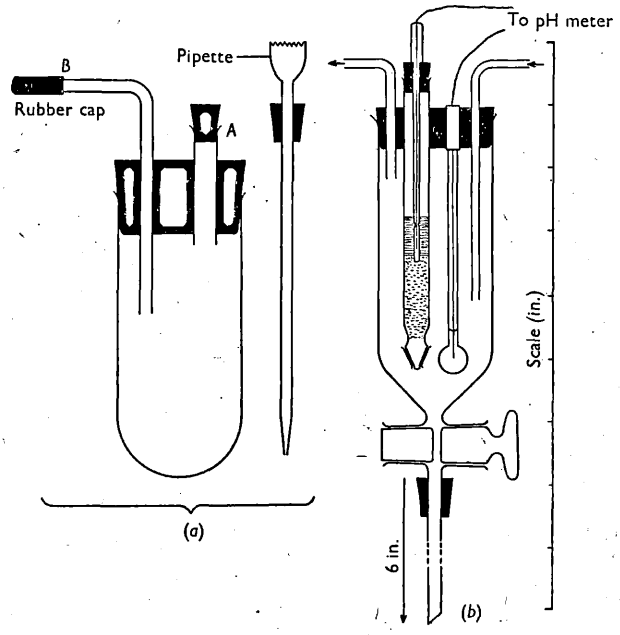

Fig. 1. Apparatus used for the determination of titration curves.

The first of these errors will be appreciable in the absence of salt, and may in part be the cause of the difference in the shape of the curves obtained with and without salt. In the presence of 0·5 M-NaCl it is negligible (Retterova, private communication). Since the second source of error partially compensates for the third, no correction was made for it. As the volume of solution is large compared with the amount of collagen, the net error must always be small. Assuming the collagen binds 50% of water (Cheshire & Holmes, 1942), the error at pH 1 would be of the order of

muscle fibres, sweat glands, fat cells, etc. will be removed with the grain layer (Dempsey, 1946). The remaining impurities are, therefore, a small amount of reticular tissue and some elastic fibres, the presence of which should not greatly affect the results obtained.

The total N of the present collagen preparation is rather higher than the value generally quoted for skin collagen, but is the same as that obtained by

Table 2. *Analyses of collagen*

	N as % protein N	g./100 g. collagen	mmol./g. collagen
Total N	—	18·6	—
Amide N	3·5	0·66	0·47
Amino N	2·5	0·46	0·33
Methionine	0·4	0·8	0·05
Aspartic acid	3·6	6·3	0·47
Glutamic acid	5·8	11·3	0·77

Bergmann & Stein (1939) for ox-hide tendon prepared in a similar manner. Earlier values for skin collagen have been determined on material which has had some treatment (alkaline or enzymic) likely to lead to loss of nitrogen from amide or guanidino groups. The amide N is considerably higher than values quoted for gelatin (Dakin, 1920; Chibnall, 1942), presumably because gelatin is usually made under conditions of alkali treatment which favour the breakdown of the amide groups (Highberger & Stecker, 1941; Ames, 1944). The amino N is of the same order as values obtained by Rutherford, Harris & Smith (1937) and Kanagy & Harris (1935) for hide powder.

The values for the dicarboxylic acids are considerably higher than those obtained by Dakin (1920) for gelatin, and Schneider (1940) for collagen, and are of the same order as values recently obtained for gelatin by microbiological methods (Hac & Snell, 1945; Stokes & Gunness, 1945; Lewes & Olcott, 1945; Hac, Snell & Williams, 1945; Hier, Graham, Friedes & Klein, 1945). Gale (1945), using a specific decarboxylase, found an average value of 10% glutamic acid for this and other similar samples of collagen prepared by the present authors. This value is likely to be low owing to racemization of the glutamic acid during hydrolysis (Gale, 1945).

The methionine content of the present collagen is lower than that quoted by Baernstein (1932) for gelatin, but is in good agreement with recent microbiological determination on gelatin: Horn, Jones & Blum (1946), 0·78%; Lyman, Moseky, Butler, Wood & Hale (1946), 0·88 and 0·83%.

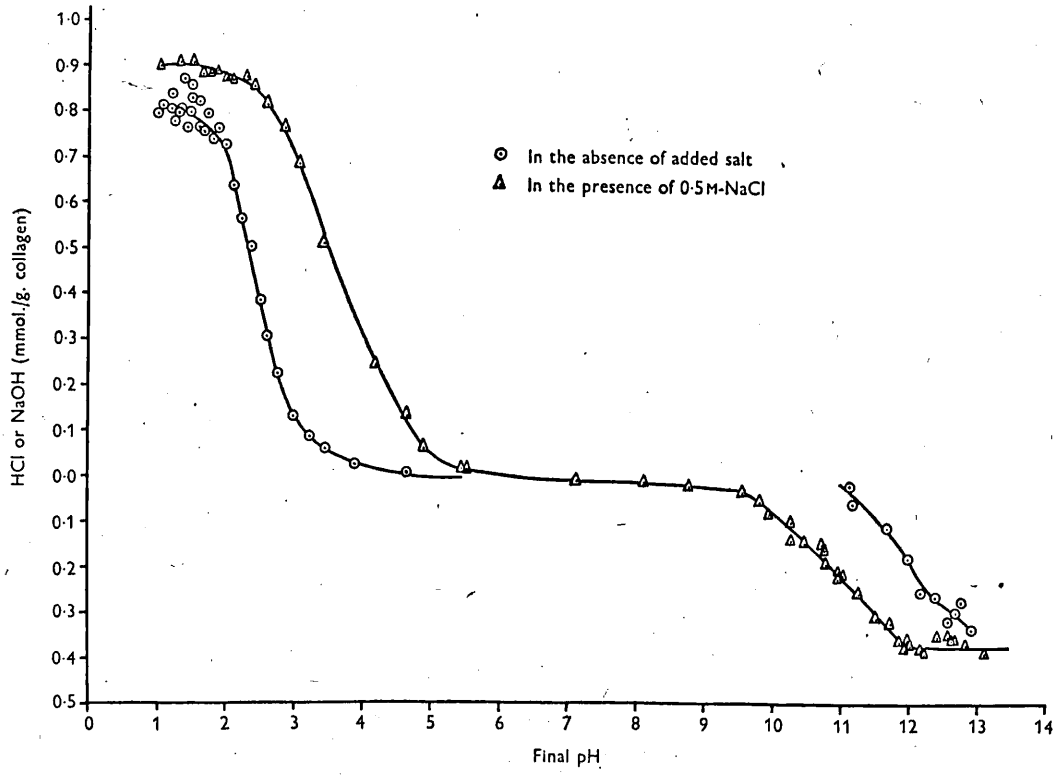

Fig. 2. Titration curves of collagen with and without sodium chloride.

Titration curves

As in previous titration curves of collagen and other fibrous proteins, the curve in the absence of salt shows a broad isoelectric range in which no

combination with acid or alkali takes place, and the acid and alkaline portions of the curve are shifted to lower and higher pH values respectively (Fig. 2). In the presence of sodium chloride, however, there is a definite isoelectric point at pH 6·6–6·8, and the curve approaches that of a soluble protein. The curve shows clearly defined acid and base-binding maxima of 0·90 and 0·36 mmol./g. of collagen at pH 1·5 and 12·5 respectively.

In contrast to the findings of Theis & Jacoby (1941*c*; 1942*b*), who used collagen which had been given a preliminary alkaline treatment, the acid-binding maximum is greater in the presence of salt than in its absence, and there is little indication that the two curves would meet if extended to lower pH values. There is also an indication that maximum base binding is less in the absence of salt.

Previous titration curves of collagen, all of which were determined on collagen which had received either an alkaline or enzymic treatment, show isoelectric points between 5·4 and 7·9 (Theis & Jacoby, 1940, 1941*a*, *b*, *c*; 1942*a*, *b*; 1943*a*, *b*; Atkin, 1937), and in those curves where definite maxima can be distinguished (Theis & Jacoby, 1941*c*, 1942*a*, 1943*a*, *b*), an acid-binding capacity of 0·85–0·90 mmol./g., and a base-binding capacity of 0·38–0·45 mmol./g. The curves of alkali-treated collagen all show an appreciable base-binding capacity in the pH range 6–8, which is absent in the present curve; this is also characteristic of titration curves of gelatin, and may be attributed to increase in the number of carboxyl groups resulting from hydrolysis of amide groups. The present curve can be divided into three portions, pH 1·5–4·9, 4·9–9·6, and 9·6–12·5, and from consideration of the probable pK's of the groups involved, may be interpreted as indicated in Table 3.

It is not possible to distinguish separate sections of the curve due to titration of the imidazole and α-amino groups, as these are present in small amounts and do not differ greatly in pK (imidazole, pK 5·5–6·5; α-amino, pK 7·5–8·5 (Cohn & Edsall, 1943)). Since collagen contains a high proportion of proline and hydroxyproline, it is possible that there are some terminal imino as well as α-amino groups, and these will titrate in a similar pH range (imino of proline, pK 9·7; imino of hydroxyproline, pK 10·1 (Cohn & Edsall, 1943)). It was also not possible to assign any portion of the curve to titration of phenolic hydroxyl groups, and in view of the small amount of tyrosine in collagen and the doubt concerning the extent to which it titrates (Fruton & Lavin, 1939; Steinhardt, 1939; Neuberger, 1943) it has been omitted from the analysis of the curve.

There is no indication that the guanidino group is titrating in the pH range covered, and the pK of this group in collagen must be greater than 14. A similar observation has been made by Lichtenstein (1940) in connexion with the titration curve of gelatin.

DISCUSSION

As far as is known to the present authors, the figures for total N, amide N and amino N are the first to be reported on skin collagen which has received no alkaline or enzymic treatment, and it is not surprising, therefore, that the values for the first two are higher than those generally quoted for collagen.

The free amino N as determined by the Van Slyke method and the number of ε- and α-amino groups deduced from the titration curve (see Table 3) are in close agreement, but appreciably less than the value indicated by determination of the lysine and hydroxylysine in the basic amino-acid fraction of

Table 3. *Analysis of titration curve*

Groups titrating	Method of calculation	pK from curve	Amount present (mmol./g.) From curve	From analysis
(*a*) Total basic groups	Titration from 1·5 to isoelectric point	—	0·90	0·94
(*b*) Imidazole	Titration from 4·9 to 9·6	7·5	0·07	0·05
Imino				—
α-Amino				—
(*c*) ε-Amino*	Titration from 9·6 to 12·5	11·0	0·34	0·39 (0·33 (Van Slyke))
(*d*) Free carboxyl	Titration from 1·5 to 4·9	3·5	0·87	0·79
(*e*) α-Amino + imino	(*b*) − 0·05 mmol. imidazole groups	—	0·02	—
(*f*) Guanidino	(*a*) − (*b*) − (*c*)	>14	0·49	0·51
(*g*) Amide	From analysis	—	—	0·47
(*h*) Dicarboxylic acids	(*d*) + (*g*) − (*e*)†	—	1·32	1·26

* Including the side-chain NH_2 group of hydroxylysine. The pK of the amino group not α to the carboxyl group in hydroxylysine is 9·50 as compared with 10·3 for the ε groups of lysine under the same conditions (Van Slyke, Hiller, MacFadyen, Hastings & Klemperer, 1940), and it may be assumed to titrate in approximately the same range as the ε-amino groups of lysine.

† It is assumed that terminal carboxyl groups are equivalent to α-amino + imino groups.

the hydrolysate (Macpherson, 1946). It is possible that in the intact protein some of the ε-amino groups are bound into the polypeptide chain or for some reason not available for reaction, or alternatively the analytical figure may be too high. The lysine N was determined by difference (total N of basic fraction minus arginine, histidine and hydroxylysine N), and hence is subject to a cumulative error.

The analytical results obtained in this investigation, together with those previously obtained (Chibnall, 1946; Macpherson, 1946; Rees, 1946; Tristram, 1946) on the same sample of collagen, are given in Table 4.

Table 4. *Composition of collagen*

Amino-acid	(1) N as % protein N	(2) As g./100 g.	(3) As g. residues/ 100 g.	(4) As mmol./g.	(5) Minimum mol. wt. calc. from data in (1)	(6) Assumed number of residues	(7) Apparent minimum mol. wt. calc. from (5) and (6)
Total N	—	18·6 *a*	—	—	—	—	—
Amino N	2·5 *a*	0·46	—	0·33	—	—	—
Glycine	26·3 *c*	26·2	19·9	3·50	286	136	38,880
Alanine	8·0 *b*	9·5	7·6	1·06	941	41	38,580
Leucine Isoleucine	3·2 *b*	5·6	4·8	0·42	2,350	17	39,950
Valine	2·2 *b*	3·4	2·9	0·29	3,420	11	37,620
Phenylalanine	1·9 *b*	~~4·2~~ 2·5	3·7	0·25	3,960	10	39,600
Tyrosine	0·6 *b*	1·4	1·3	0·08	12,540	3	37,620
Tryptophan	0·0 *d*	0·0	0·0	0·00	—	—	—
Serine	2·5 *b*	3·4	2·7	0·33	3,010	13	39,130
Threonine	1·5 *b*	2·4	2·0	0·20	5,020	8	40,160
Cystine	0·0 *e*	0·0	0·0	0·00	—	—	—
Methionine	0·4 *a*	0·8	0·7	0·05	18,820	2	37,640
Proline	9·9 *b*	15·1	12·7	1·32	760	51	38,760
Hydroxyproline	8·0 *c*	14·0	12·1	1·07	941	41	38,580
Lysine	4·7 *b*	4·5	4·0	0·31	3,200	12	38,400
Hydroxylysine	1·2 *b*	1·3	1·1	0·08	12,560	3	37,680
Arginine	15·3 *b*	8·8	7·9	0·51	1,969	20	39,380
Histidine	1·2 *b*	0·8	0·7	0·05	18,820	2	37,640
Aspartic acid	3·6 *a*	6·3	5·5	0·47	2,092	19	39,750
Glutamic acid	5·8 *a*	11·3	10·0	0·77	1,297	30	38,910
Amide N	3·5 *a*	0·66	—	0·47	2,157	18	38,740
Total found	99·8	119·00*	99·6*	10·76*	—	419	38,730. (mean)

* Excluding amide N.

(*a*) Determined by present authors.
(*b*) Determined by Chibnall and collaborators on the same sample of collagen.
(*c*) Bergmann & Stein (1939).
(*d*) Block & Bolling (1945).
(*e*) Baernstein (1930).

The values obtained by the present authors for the dicarboxylic acids are 50% higher than the earlier values of Dakin (1920), and, although probably still an underestimate, are the best values at present available for collagen. (Consden *et al.* (1948) report 91 and 96% recoveries of glutamic and aspartic acids, respectively, from synthetic mixtures simulating wool.) If a correction based on the results of Consden *et al.* (1948) on wool is applied, the total dicarboxylic acid content agrees well with that deduced from the titration curve.

With the exception of the ε-amino groups of lysine, the values deduced from the titration curve are in agreement with those obtained by analysis, and there is no indication that any of the other reactive side-chain groups of the basic or dicarboxylic acids are bound, or that there is any appreciable number of terminal amino or carboxyl groups.

From a survey of the literature, it is evident that of the remaining amino-acids, tryptophan and cystine are absent, but that glycine and hydroxyproline are present in considerable amounts. Values for these last two have been selected from the literature to complete the attempted survey of the amino-acid composition of collagen given below. Of these, the figure for hydroxyproline is most likely to be in error; the method used is not entirely reliable and the determination was made on gelatin.

When the composition of collagen was considered by Braybrookes (1939) and by Bowes (1943) the analysis was incomplete. The higher figures now obtained for some of the amino-acids, notably phenylalanine and the dicarboxylic acids, and the addition of valine, threonine, serine, and hydroxy-

lysine to the list of amino-acids present in appreciable amounts, have now made up the deficiency. Using the values given in Table 4, it is now possible to account for over 99 % of the total N of collagen (see col. 1), and the sum of the amino-acid residues (col. 3) approaches 100. The analysis of collagen is, therefore, virtually complete. Although the values of some of the amino-acids may be adjusted slightly as a result of improved methods of analysis; it is unlikely that any great changes in the values will be made, or that other amino-acids will be found to be present in important amounts.

Calculation of the minimum molecular weight from the data in cols. 1, 5 and 6 gives a value of about 39,000. Earlier calculations from the composition of gelatin have given figures of 10,300 (Jordan Lloyd, 1920), 10,000–12,700 (Cohn, Hendry & Prentiss, 1925), and 34,500 (Atkin, 1933).

The mean residue weight calculated by summation from the data in Table 4

$$\frac{\Sigma \text{ wt. of amino-acid residues/100 g. protein}}{\Sigma \text{ g. residues/100 g. protein}}$$

is 92·6, and the same value is obtained by calculation from the N distribution (Chibnall, 1942). From X-ray data and the density of dry gelatin Astbury (1940) obtained a value of 94 for the average residue weight in gelatin. Taking 39,000 as the mean molecular weight and 92·6 as the average residue weight, the number of residues/molecule is 421, of which 419 have been identified (col. 6, Table 4).

SUMMARY

1. Collagen has been prepared from ox hide with the minimum of chemical treatment. The total N and amide N contents of the collagen are higher than those previously reported. The glutamic and aspartic acid contents are nearly double the values quoted by Dakin (1920) for gelatin.

2. The titration curve of this collagen has been interpreted in terms of the titratable groups, and the values deduced are, in general, in agreement with those obtained by direct analysis, and indicate that the side-chain groups of the basic and dicarboxylic acids are free, and that the number of terminal groups is small.

3. It is now possible to account for over 99 % of the nitrogen in collagen, and it is considered that it is unlikely that any further amino-acids will be found to be present in important amounts.

4. The minimum molecular weight calculated from the analytical figures is 39,000, and the average residue weight calculated both by summation and from the nitrogen distribution is 92·6.

In the course of this and other work the authors have had occasion to refer to the various determinations of the amino-acids in gelatin and collagen which have been made at different times, and it has been thought useful to put these on record. Copies of this compilation may be obtained from the Librarian, British Leather Manufacturers' Research Association.

The authors wish to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper, the Director of the Wool Industries Research Association, in whose laboratories the determination of the dicarboxylic acids was carried out, and Dr R. Consden for advice and assistance in these determinations. They also wish to thank Dr E. F. Gale for determinations of the glutamic acid content of various samples of collagen.

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The Effect of Alkalis on Collagen

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An essential process in the conversion of hides and skins to most types of leather is the removal of the hair or wool by treatment in suspensions of lime and sodium sulphide, usually in the cold, for periods varying from a few hours to several weeks.

Although several workers have investigated the effect of mild alkaline treatment on collagen (Marriott, 1928, 1931; Küntzel & Phillips, 1933; Kubelka & Knöedel, 1938; Highberger & Stecker, 1941; Ames, 1944), each has dealt only with one or two of the possible changes which may occur, and in only one instance (Marriott, 1931) has a collagen been used which has had no previous alkaline or enzymic treatment.

The present investigation has been carried out, therefore, on a collagen which has received no previous chemical treatment likely to cause chemical modification (see Bowes & Kenten, 1948), with the aim of obtaining more precise information regarding possible changes occurring during mild alkaline treatment.

EXPERIMENTAL

Alkali treatment of collagen. Collagen prepared as previously described (Bowes & Kenten, 1948) was cut into cubes about 1 cm. square, and 100 g. of this material, together with 310 ml. of water, 1·55 g. of $Ca(OH)_2$, and 90 ml. of N-NaOH were incubated in a sealed flask at 20°. A preliminary experiment showed that these quantities gave an initial OH^- concentration of 135 m-equiv./l. (pH 13·3 at 20°), and a final value of 68 m-equiv./l. (pH 13·0 at 20°). After 14 days the flask was cautiously opened to avoid loss of NH_3, sufficient HCl added to bring the contents to pH 4·5–5·0, the flask resealed and left at 20° for 3 days. Two batches of collagen (I and II) were treated in this way.

The collagen was filtered off, washed in running water for several hours, treated repeatedly with acetic acid pH 3 containing 1·5% NaCl, exhaustively washed with water at pH 8, finally with water at pH 6, then dehydrated with acetone. The pH of the last wash water was 5·5. (A little toluene was added to all wash waters to inhibit the growth of micro-organisms.)

An appreciable amount of protein matter (solubilized collagen) was precipitated on neutralization. This material was filtered off with the collagen and removed during the washing in running water.

The total N, amino N, NH_3 and urea were determined in the solution in which the collagen had been treated. It was assumed that the solution held by the collagen was of the same composition as the external solution, and that the total volume was equal to that of the solutions added plus the water held by the original collagen, and the results were calculated accordingly. The total N, amino N, amide N, arginine, total hydroxy acids (serine + threonine + hydroxylysine), and the titration and swelling curves of the collagen, were also determined. All results are expressed on moisture- and ash-free collagen. Since the total N of the alkali-treated collagen is lower than that of the original collagen, the results expressed as a percentage of the total N may be misleading, and they have therefore been recorded also as mmol./g. protein.

Analysis. Total N, amide N and amino N were determined as previously described (Bowes & Kenten, 1948). Arginine was determined by precipitation with flavianic acid (Vickery, 1940). The value obtained (8·3%) was rather lower than that of 8·8% found for the same collagen sample by Macpherson (1946), who used a colorimetric method following the separation of the basic amino-acids by electrodialysis.

Hydroxyamino-acids. The total hydroxyamino-acids (serine + threonine + hydroxylysine) were determined by the periodate method as described by Rees (1946), except that the periodate oxidation and subsequent distillation of the NH_3 were carried out in a micro-Kjeldahl apparatus. The following solutions were successively added to the micro-Kjeldahl apparatus, while preserving a liquid seal at the ground-glass stopper: 5 ml. of hydrolysate, 5 N-NaOH to adjust the pH to 7, N-H_2SO_4 (sufficient to give a final pH of 7·0 after addition of other reagents), 1·5 ml. of $NaAsO_2$ solution, 6 ml. of phosphate buffer pH 7·2, 1 ml. of 0·5 M-HIO_4, 3 ml. water. The contents of the micro-Kjeldahl flask were thoroughly mixed after each addition by warming the steam flask, and thereby causing a stream of air bubbles to pass through the solution. The NH_3 (preformed and periodate) was distilled off from 5 ml. of borate buffer pH 12·0. The preformed NH_3 was determined by a separate distillation of 5 ml. of hydrolysate at pH 7·0 from 5 ml. of borate buffer.

Other determinations. Ammonia in solution was determined by addition of excess K_2CO_3 and aerating into standard acid; urea by conversion to NH_3 by the action of urease followed by the same procedure (Van Slyke & Cullen, 1914). The urea NH_3 was then calculated by difference. It was necessary to use twice as much urease as that employed by Van Slyke & Cullen (1914) in order to obtain satisfactory results; this was probably due to inhibition of the urease by the high salt concentration of the solution (cf. Conway, 1947). The amount of urea was small compared with that of NH_3, and tests showed that under the present experimental conditions the method gave an accuracy of ±10%.

Titration and swelling curves. The titration curve of the alkali-treated collagen (batch I) was determined in the presence of 0·5 M-NaCl as previously described (Bowes & Kenten, 1948). For the swelling curves, 0·5 g. samples of air-dry collagen were placed in 100 ml. of solution containing varying amounts of HCl and NaOH at 20°. After 3 days, a sample of liquid was withdrawn for pH determination, and the pieces of collagen lightly blotted and weighed. The vessels used were the same as those employed for the titration curve, and the same precautions were taken to avoid contamination with CO_2. The water content, expressed as a percentage of the moisture and ash-free collagen, was plotted against the final pH value of the solution.

RESULTS

Analytical

There is good agreement between the results on the two separate batches of treated collagen, indicating that the results obtained are reproducible (see Table 1).

Although the treatment given was comparatively drastic, the collagen only decreased in weight by about 5%, and was unchanged in appearance. Approximately half the amide N was liberated during 14 days' treatment, and a corresponding amount of ammonia was found in the solution; there

Table 1. *Analyses of collagen, alkali-treated collagen and of collagen dissolved by alkaline solution*

	Collagen					Solution		
		Alkali-treated		Increase or decrease				
	Untreated	Batch I	Batch II	Batch I	Batch II		Batch I	Batch II
Ash (%)	0·03	0·01	0·10	—	—	—	—	—
Water (%)	22·3	23·7	23·8	—	—	—	—	—
Total N (%)	18·6	18·2	18·1	−0·4	−0·5	Total N (mg./g.)	9·0	8·1
Amino N (as % TPN)	2·48	2·77	2·94	—	—	Amino N (mg./g.)	4·3	4·3
Amino N (mmol./g.)	0·33	0·36	0·38	+0·03	+0·05	—	—	—
Amide N (as % TPN)	3·54	1·69	1·85	—	—	Ammonia N (mg./g.)	3·46	3·36
Amide N (mmol./g.)	0·47	0·22	0·24	−0·25	−0·23	Ammonia N (mmol./g.)	0·25	0·26
Arginine N (as % TPN)	14·45	14·17	14·24	—	—	Urea N (mg./g.)	0·45	0·42
Arginine N (mmol./g.)	0·48	0·46	0·46	−0·02	−0·02	Urea (mmol./g.)	0·016	0·015
Periodate NH_3 (as % TPN)	4·54	4·45	—	—	—	—	—	—
Periodate NH_3 (mmol./g.)	0·60	0·58	—	−0·02	—	—	—	—

TPN = total protein nitrogen.

was no indication that an appreciable amount of ammonia was derived from any other source. The amount of ammonia formed was almost identical with the maximum value obtained by Highberger & Stecker (1941). Further treatment for 7 days under similar conditions, however, lowered the amide N by another 0·08 mmol. to 0·16 mmol./g., with the production of 0·06 mmol. of ammonia.

The small decrease in the arginine content is less than that found by Theis & Jacoby (1941) in similar experiments, and the urea found in the solution was also less than that found by Highberger & Stecker (1941) under corresponding conditions. It appears probable that the urea arises from conversion of the arginine to ornithine residues (cf. Hellermann & Stock, 1938; Warner, 1942*a*); the decrease in arginine content, however, is rather greater than can be accounted for as urea, indicating some other type of breakdown. The alkali-treated collagen, when tested for the presence of citrulline by the method of Fearon (1939), gave a very faint pink colour, the original none at all.

Within experimental error, the ammonia formed by the action of periodate on the untreated and alkali-treated collagen is the same, indicating a negligible destruction of the hydroxyamino-acids by the alkaline treatment. The hydroxyamino-acids of the collagen used in this investigation have also been determined by Rees (1946), who obtained a value of 4·57 % for the periodate NH_3 (expressed as a percentage of the total protein N), in good agreement with the present determination of 4·54 %.

The ammonia and urea in the solution account only for about half of the total N present. The figure for the amino N of the solution is likely to be too high owing to the presence of ammonia (25–30 % of which may be returned as amino N (Warner, 1942*b*; Frost & Heinsen, 1945)), but the results suggest that this soluble N must be present as amino-acids or small peptides. It may be observed that the sum of the ammonia and urea N in solution is approximately equal to the decrease in the total N of the collagen.

The amino N of the alkali-treated collagen was only slightly higher than that of the original collagen (this small increase could be accounted for by the conversion of arginine to ornithine), and, even allowing for the fact that because of the high proline and hydroxyproline content of collagen one out of every four peptide links involves an imino and not an amino group, the alkaline treatment has apparently caused little breakdown of the polypeptide chains of the remaining insoluble collagen.

Titration curves

At pH values between 5 and 10, a longer time was required to reach equilibrium with alkali-treated collagen than with the original collagen. There was rather more soluble nitrogen at high pH values, and rather less at low pH values than with the original collagen (see Table 2).

Table 2. *Nitrogen in solution at different* pH *values after steeping for* 3 *days*

(Results expressed as mg. N/g. moisture- and ash-free collagen.)

Collagen				Alkali-treated collagen			
pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N
1·16	0·85	12·86	1·44	1·18	0·48	—	—
1·32	0·77	12·71	1·08	—	—	12·77	2·15
1·51	0·69	12·66	1·21	1·42	0·36	12·69	2·05
1·64	0·70	12·59	1·28	1·82	0·34	12·54	2·26
—	—	12·46	1·04	—	—	12·39	1·26
—	—	—	—	—	—	12·20	1·45

The titration curve of the alkali-treated collagen differs in a number of respects from the curve of the original collagen, especially in the alkaline range (see Fig. 1). The isoelectric point is shifted from pH 6·7 to 5, the acid binding is slightly increased, and on the alkaline side of the isoelectric point there is a large increase in the number of groups titrating between pH 5 and 9, and a corresponding increase in the base binding capacity. The large increase in the groups titrating between pH 5 and 9 may be attributed to an increase in the number of carboxyl groups arising from hydrolysis of amide groups.

The curve was analyzed in a manner similar to that of the untreated collagen (see Bowes & Kenten, 1948). It can be divided into four portions, from pH 1·5 to 5, from 5 to 7, from 7 to 9·6 and from 9·6 to 12, and to these different portions are assigned the groups shown in Table 3. Imino groups, due to breakdown of peptide links involving proline or hydroxyproline, are taken as mainly titrating between pH 7 and 9·6.

Assuming that the imidazole groups are unaffected by alkaline treatment (Plimmer, 1916), and that they titrate in the same range as in the untreated collagen, it can be deduced that there are 0·14 mmol. uncharged carboxyl groups/g., and 0·13 mmol. basic groups/g. (α-amino, imidazole or imino), titrating between the isoelectric point and pH 9·6. The assumption that the imidazole groups

are not affected may not be strictly true, since there is some evidence that gelatin contains less histidine than collagen (Chibnall, 1946). The difference, however, is small, and even if a corresponding decrease in histidine does occur here, it would not materially affect the calculation. The same conclusion is reached if the increase in acid-binding capacity is taken as due to increase in the number of α-amino and imino groups, again assuming that these groups titrate between pH 5 and 9·6, and that the α-amino and imidazole groups present in the original collagen remain unchanged.

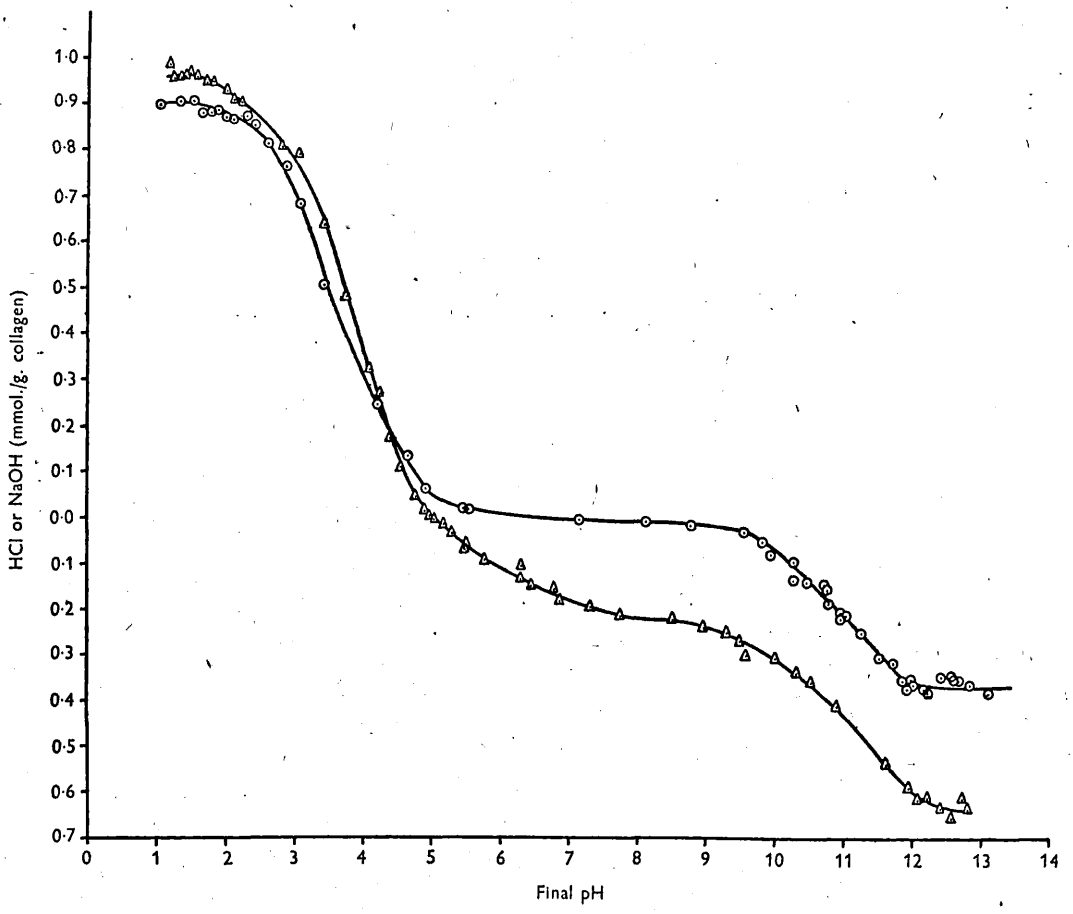

Fig. 1. Titration curves of ox-hide collagen before (circles) and after (triangles) treatment with alkali.

Table 3. *Analyses of titration curves*

(Results expressed as mmol./g.)

Group	Method of calculation	Original collagen		Alkali-treated collagen	
(*a*) Charged carboxyl	Titration from pH 1·5 to 5	0·87		0·96	
(*b*) Uncharged carboxyl + imidazole	Titration from pH 5 to 7	0·03	0·07	0·17	0·27
(*c*) Imidazole, α-amino + imino	Titration from pH 7 to 9·6	0·04		0·10	
(*d*) ε-Amino	Titration from pH 9·6 to 12·5	0·34		0·36	
(*e*) Uncharged carboxyl	(*b*) − 0·03 mmol. imidazole groups titrating in this range	0		0·14	
(*f*) Total carboxyl	(*a*) + (*e*)	0·87		1·10	
(*g*) α-Amino + imino	(*c*) − 0·02 mmol. imidazole groups titrating in this range	0·02		0·08	
(*h*) Guanidino	(*a*) + (*e*) − (*b*) − (*c*) − (*d*)	0·49		0·47	
(*i*) Amide	From analysis	0·47		0·22	
(*j*) Dicarboxylic acids	(*f*) + (*i*) − (*g*)*	1·32		1·24	

* It is assumed that terminal carboxyl groups are equivalent to the free α-amino and imino groups.

If these conclusions are correct, there is an apparent decrease in the dicarboxylic acids, which suggests that there may have been specific loss of these amino-acids in the fraction of the collagen rendered soluble by the alkaline treatment.

The decrease in arginine content calculated from the titration curve agrees well with that found by analysis (Table 1), and the small increase in groups titrating between pH 9·6 and 12 is in accordance with the hypothesis that the arginine is converted to ornithine (pK 10·76) (Cohn & Edsall, 1943). There is no indication of any decrease in the number of ϵ-amino groups.

Swelling curve

The uptake of water by the collagen at different pH values before and after treatment with alkali

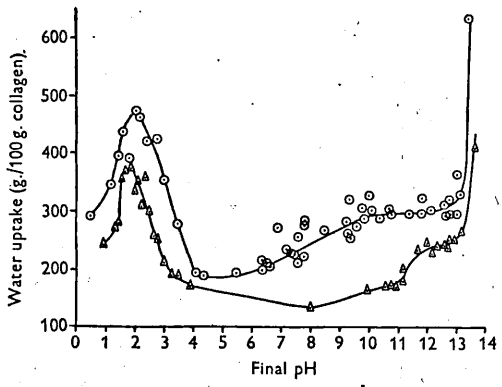

Fig. 2. The water uptake of ox-hide collagen before (triangles) and after (circles) treatment with alkali.

is shown in Fig. 2. After exposure to the acid or alkaline solutions, the pieces of alkali-treated collagen had a roughened appearance especially at high or low pH values. This was probably an indication of the beginning of disintegration. At all pH values the water uptake is appreciably increased by the alkaline treatment, and on the alkaline side of the isoelectric point the shape of the curve is appreciably different from that of the original collagen.

Analysis of samples of skin collagen after alkaline processing ('liming')

The total N, amino N and amide N were determined in a number of skins which had been treated commercially with calcium hydroxide and sodium sulphide to remove the hair ('limed'). The titration curves and arginine contents of three samples were also determined.

Table 4 shows that, with the exception of the sheep-skin collagens, the total N of all the samples was of the same order as that of the alkali-treated ox-hide collagen. The amide-N values varied from 0·25 to 0·47 mmol./g., the sheep skins on the whole having the lowest values. These skins had been treated with a commercial preparation of trypsin ('bating') and with sulphuric acid (approx. 1%) and sodium chloride ('pickling'), and this is probably the cause of the lower values. In a number of cases, the amino N of the commercially treated samples is lower than that of the ox-hide collagen.

The titration curves of sheep skins 1, 2, 3 differ from one another and from the curves for ox-hide collagen, especially in the pH range 6–9.

The curves are more difficult to interpret in terms of the reactive groups than those of the ox-hide collagen. The various sections of the curve, especially those due to titration of unionized carboxyl, imidazole, α-amino and imino groups tend to merge into one another. Treating the curves similarly to that of the alkali-treated collagen, the analyses given in Table 5 were deduced.

Table 4. *Total* N, *amide* N *and amino* N *of collagen of some commercially treated skins*

Source of collagen	Total N (%)	Amide N (%)	Amide N (mmol./g.)	Amino N (%)	Amino N (mmol./g.)
Ox-hide collagen (no alkaline treatment)	18·6	0·66	0·47	0·46	0·33
Sheep skins 1	17·3	0·42	0·30	—	—
2	17·1	0·42	0·30	0·59	0·42
3	17·2	0·35	0·25	0·50	0·36
4	17·4	0·50	0·36	0·45	0·32
5	17·3	0·49	0·35	0·49	0·35
Calf skins 1	18·2	0·56	0·40	—	—
2	18·2	0·52	0·37	—	—
3	18·0	0·55	0·39	0·42	0·30
Ox hide 1	18·1	0·66	0·47	0·46	0·33
2	18·1	0·42	0·30	0·45	0·32
3	18·3	0·46	0·33	0·48	0·34
4	18·1	0·57	0·41	0·42	0·30

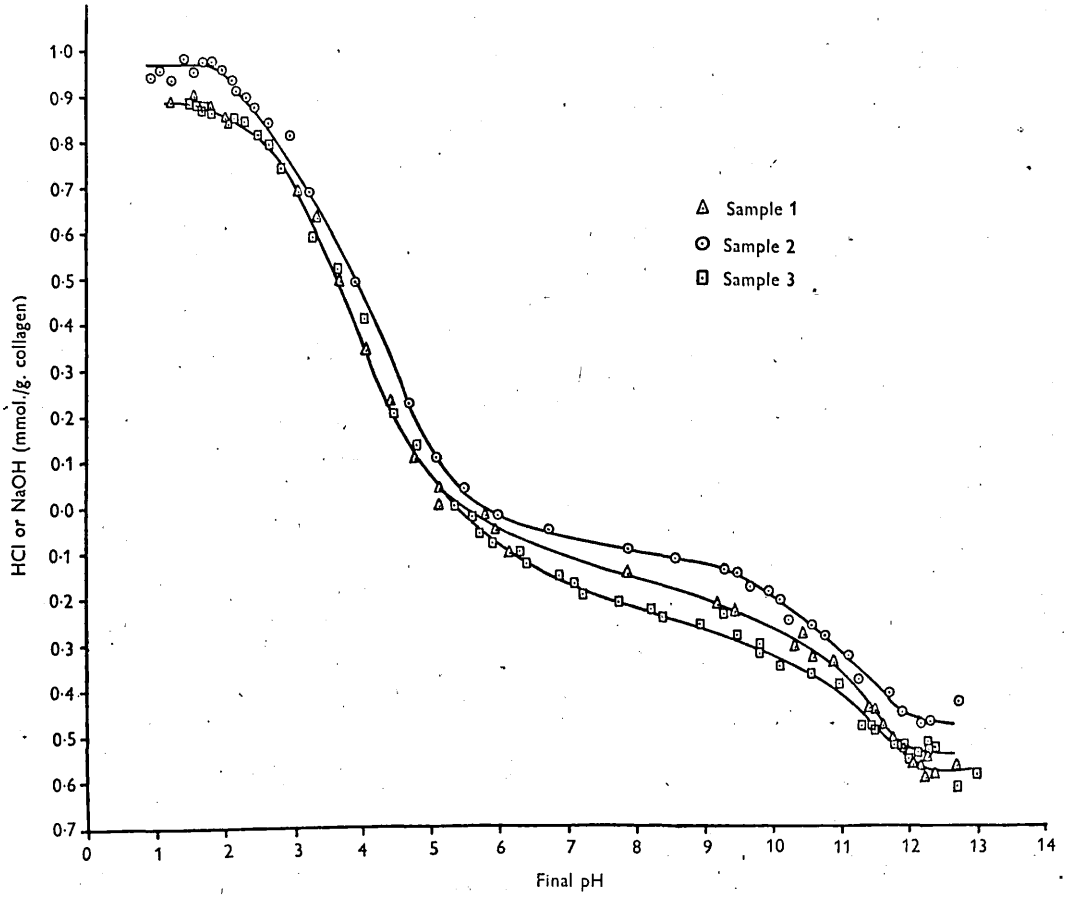

Fig. 3. Titration curves of commercially treated sheep skins.

Table 5. *Analyses of titration curves of alkali-treated sheep-skin collagens*

(Results expressed as mmol./g.)

Group	Method of calculation	Alkali-treated sheep-skin collagen		
		Sample 1	Sample 2	Sample 3
(*a*) Charged carboxyl	Titration from pH 1·5 to isoelectric point	0·89	0·97	0·88
(*b*) Uncharged carboxyl + imidazole	Titration from isoelectric point to pH 7	0·10	0·06	0·16
(*c*) Imidazole, α-amino + imino	Titration from pH 7 to 9·6	0·14	0·10	0·13
(*d*) ε-Amino	Titration from pH 9·6 to 12·5	0·34	0·32	0·26
(*e*) Uncharged carboxyl	(*b*) − 0·03 mmol. imidazole groups titrating in this range	0·07	0·03	0·13
(*f*) Total carboxyl	(*a*) + (*e*)	0·96	1·00	1·01
(*g*) α-Amino + imino	(*c*) − 0·02 mmol. imidazole groups titrating in this range	0·12	0·08	0·11
(*h*) Guanidino	(*a*) + (*e*) − (*b*) − (*c*) − (*d*)	0·38	0·52	0·46
(*i*) Amide	From analysis	0·30	0·30	0·25
(*j*) Dicarboxylic acids	(*f*) + (*i*) − (*g*)*	1·14	1·22	1·15

* It is assumed that terminal carboxyl groups are equivalent to terminal α-amino and imino groups.

DISCUSSION

Considering the comparative severity of the alkaline treatment, the chemical modification of the collagen is comparatively small. Apart from the solubilization of about 5 % of the collagen, the only reaction taking place to any appreciable extent is the hydrolysis of amide groups. Since the loss of amide groups increases with time, it appears probable that all the amide N would be liberated if the treatment were sufficiently prolonged.

The small decrease in arginine content and the presence of urea in solution indicates a breakdown to ornithine, as postulated by Hellermann & Stock (1938) and Warner (1942*a*). A positive test for citrulline in the alkali-treated collagen indicates that the conversion of arginine to citrulline and ammonia also takes place to a very small extent (see Warner, 1942*b*). The amount of ammonia arising from arginine is negligible compared with that due to deamidation.

Nicolet, Shinn & Saidel (1942) have shown that treatment of silk fibres with boiling 0·1 M-alkali for 1–2 hr. causes partial destruction of serine and threonine, producing an approximately equivalent number of amide groups. There is no evidence that such a reaction occurs even to a limited extent when collagen is treated with alkali in the cold for periods up to 14 days.

The ornithine produced by modification of the guanidino groups could account for the small increase in the base bound between pH 9·6 and 12·5, and for the greater part of the increase in free amino N. On this basis, there can be little, if any, hydrolysis of peptide links involving amino groups in the alkali-treated collagen, and the increase in acid-binding capacity (0·06 mmol./g.) must presumably be due to release of basic groups other than amino groups. It is possible that these are imino groups, derived from hydrolysis of peptide links involving proline or hydroxyproline. Whilst this would suggest that such peptide links are more alkali-labile than those involving amino groups, Gordon, Martin & Synge (1943), from a study of the partial acid hydrolysate of gelatin, inferred that they were resistant to acid hydrolysis at 37°.

Although there is only a small increase in the number of terminal groups in the alkali-treated collagen, an appreciable amount (5 %) of the collagen was rendered soluble by the treatment. Some of this was presumably in the form of large polypeptides, since it was precipitated on neutralization of the solution; peptides which did not precipitate probably account for nitrogen in the solution in excess of that due to ammonia and urea. The amino-N figure for the solution suggests that this fraction must be in the form of amino-acids or small peptides. Evidence deduced from the titration curve suggests that this soluble fraction is rich in the dicarboxylic acids. Martin (1946) has recently demonstrated the presence of aspartylglutamic, glutamylglutamic, and glutamylaspartic acids in the partial hydrolysate of wool, and if these two dicarboxylic acids also occur together in collagen, breakdown could readily lead to their specific loss.

Consideration of the swelling curves indicates that physical as well as chemical changes take place during the alkaline treatment, for the changes in the curve can only be partially accounted for by changes in the reactive groups. The water uptake in the isoelectric range is increased, the increase in water uptake in acid solutions is greater than corresponds to the increase in basic groups, and the increase between pH 7 and 9 can only partially be accounted for by the titration of uncharged carboxyl groups liberated by hydrolysis of amide groups. The cause of the shift in the pH at which water uptake in alkaline solutions reaches a maximum from 12·5 to 9·5 is not clear.

Examination of the commercially treated skins indicates that similar changes have taken place; the total N and amide N of these were lower than those of the original ox-hide collagen, but the amino N was in some cases lower, instead of higher as might have been expected from the laboratory experiment. It is possible that this may be due to bacterial deamination at some stage in the processing of the skins (Stuart, 1946). The total N of the sheep skins was particularly low, and is not accounted for by the lower values of the amide N and arginine N as compared with the original ox-hide collagen, which suggests the possibility that the composition of sheep-skin collagen may differ from that of ox hides.

SUMMARY

1. Hydrolysis of amide groups is the main reaction taking place when collagen is treated with alkali at pH 13·0. A small number of the guanidino groups is converted to ornithine and urea, and a still smaller fraction to citrulline and ammonia. There is a small increase in the total number of basic groups and in the number of free amino groups; it is suggested that some peptide links involving proline and hydroxyproline are broken. The hydroxyamino-acids, serine, threonine and hydroxylysine, are unaffected by the treatments. Approximately 5 % of the collagen is solubilized.

2. Alkali-treated collagen swells more than the original collagen.

3. Skins treated commercially with alkaline solutions undergo changes similar to those found in laboratory experiments.

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The Effect of Deamination and Esterification on the Reactivity of Collagen

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The most important reactive groups in collagen are amino, guanidino and carboxyl, all of which are present in comparatively large numbers; few phenolic, hydroxyl and imidazole groups are present and indole groups are absent (Bowes & Kenten, 1948*a*). A study has now been made of the effect of modification of the amino, guanidino and carboxyl groups on the acid- and base-binding capacity, and on the swelling of collagen. The combination of the modified collagens with vegetable tannins, basic chromium salts and formaldehyde has also been investigated primarily because of the importance of these three classes of compound in the conversion of collagen to leather. Moreover, the fixation of metals by proteins is of general interest, and information concerning the factors affecting the fixation of tannins may help to throw light on the association of tannins and proteins in nature.

The amino groups of proteins can readily be converted to hydroxyl groups by the action of nitrous acid. The reaction is not entirely specific, however, since at low pH values, or if the reaction is prolonged, modification of the guanidino, imidazole, tyrosine and glutamide groups may also take place (for review of literature see Olcott & Fraenkel-Conrat, 1947). With soluble proteins, reaction with the amino groups is rapid and other reactions are probably negligible, but with fibrous proteins, such

as collagen, the reaction must be prolonged to allow time for the nitrous acid to diffuse into the interior of the fibres, and some modification of other groups probably occurs. Previous workers who have used this method with collagen did not determine the extent of specificity of the reaction (Thomas & Foster, 1926; Thomas & Kelly, 1926; Gustavson, 1926; Meunier & Schweikert, 1935; Atkin, 1937; Bowes & Pleass, 1939; Highberger & Retzsch, 1939; Wilson & Yu, 1941; Gustavson, 1943; Lollar, 1943; Chang, Yen & Chen, 1944; Theis, 1945).

Modification of the guanidino groups has received little attention and no satisfactory method appears to be available. Treatment with hypochlorite, as in the Sakaguchi reaction, has been found to destroy all the arginine present in soluble proteins, but histidine, tyrosine, tryptophan and lysine are also affected, though to a lesser extent (Sakaguchi, 1925). Only about 43% of the arginine in collagen is affected by the reaction (Highberger & Salcedo, 1940). In the present investigation, it was again found that only 40–50% of the arginine in collagen was destroyed by this treatment. Roche & Morgue (1946) have shown that the reactivity, in the Sakaguchi reaction, of the guanidino group in amino-acid derivatives is influenced by the presence of other groups, some of which, notably aliphatic hydroxyl, prevent over 90% of the guanidino groups from reacting. It is possible that the difficulty of removing more than about half the guanidino groups from the collagen is related to the same cause. Since the reaction was obviously not specific to the guanidino groups, and was accompanied by extensive general breakdown of the collagen, it was not studied in detail.

A number of the reagents which react with the carboxyl groups of proteins, e.g. diazomethane, acetic anhydride, keten, methanol, are not specific to carboxyl groups (Herriott, 1947; Olcott & Fraenkel-Conrat, 1947), and with some the conditions necessary for reaction cause breakdown of the protein structure (Fodor & Epstein, 1937). Blackburn, Carter & Phillips (1941) and Blackburn & Phillips (1944) have studied the methylation of wool with methyl sulphate and methyl halides, and conclude that the main reaction is esterification of the carboxyl groups. It appeared, therefore, that these methods were likely to be satisfactory for modifying the carboxyl groups of collagen without affecting other groups to any appreciable extent, and with minimum risk of degradation.

EXPERIMENTAL METHODS

Material

The collagen was prepared from sheepskins which had been dewooled with $Ca(OH)_2$ and Na_2S ('limed'), treated with pancreatic trypsin ('bated'), and preserved with H_2SO_4 and NaCl ('pickled'). The skins were washed free of acid and salt, dehydrated with acetone, cut into strips 1×5 cm. (the edges of the skins being discarded), brought to equilibrium with distilled water (pH *c.* 5), and finally dehydrated with acetone. The titration curves of three batches of collagen prepared as above (*A*, *B* and *C*) are given in a previous paper (Bowes & Kenten, 1948*b*); they are referred to in text and tables as collagen *A*. *B* and *C*, with the prefixes *D* and *M* to denote deaminated and methylated, respectively.

Treatment of collagen

Deamination. The method used was based on that of Thomas & Foster (1926). Collagen (100 g.) was soaked in distilled water overnight, 100 g. $NaNO_2$ then added, followed by 100 ml. glacial acetic acid. A stream of CO_2 was passed through the solution to minimize oxidation of nitrous to nitric acid and to agitate the solution. After 24 hr., a further 100 g. $NaNO_2$ and 100 ml. acetic acid were added and the reaction allowed to proceed for another 24 hr. The collagen was rinsed with water, washed to remove acid in several changes of water containing 10% (w/v) NaCl to minimize swelling, brought to equilibrium with distilled water (pH 5·0), and dehydrated with acetone. Two batches of collagen *C* (DC_1 and DC_2) and one of collagen *A* (*DA*) were treated in this way.

Methylation. Methods used were similar to those of Blackburn *et al.* (1941): (1) Dimethyl sulphate. Collagen (50 g.) was shaken with 500 ml. M-Na acetate buffer (pH 7·5) and 25 ml. methyl sulphate for 1 hr. This procedure was repeated fourteen or more times. To reduce the extreme swelling of the methylated collagen it was placed in a 10% (w/v) solution of NaCl for 20 hr., then washed in distilled water until free from Cl^-, and dehydrated with acetone. (2) Methyl bromide. The collagen was brought to equilibrium with a borate buffer at pH 9·0, dehydrated with acetone, air dried, and immersed in methyl bromide for 14 days. The latter was then allowed to evaporate, and the collagen washed as before and dehydrated with acetone. In some cases the collagen was rebuffered and treated for a further 14 days with methyl bromide. Several batches of collagen *A* (MA_1 to MA_4) and of collagen *C* (MC_1 to MC_6) were methylated; also the oxhide collagen prepared as previously described (Bowes & Kenten, 1948*a*).

Combination of modified collagen with tannic acid, mimosa tannins, chromium and formaldehyde. The modified collagen (2 g.) in the form of pieces 1 cm. square, and a piece 1×5 cm. for shrinkage-temperature determinations were soaked in water overnight and then placed in 100 ml. of the following tanning solutions adjusted to different pH values for the times stated. Approximate equilibrium should have been reached in these times:

(1) 10% (w/v) tannic acid (British Drug Houses, Ltd.) for 7 days.
(2) 10% (w/v) tannic acid + 0·5 M-NaCl for 7 days.
(3) 10% (w/v) commercial mimosa tannin extract for 7 days.
(4) 3·4% (w/v) chromic sulphate for 4 days.
(5) 0·36% (w/v) formaldehyde for 3 days.

All pH values were attained with HCl or NaOH. The tannin solutions were initially adjusted to the required pH and then subsequently twice daily during the treatment. The pH of the $Cr_2(SO_4)_3$ solutions was adjusted after 24 hr., and maintained at this pH value by frequent further additions

during the following 48 hr.; no additions were made in the last 24 hr. and the pH fell slightly during this time. The pH's of the formaldehyde solutions were initially adjusted, and then allowed to fall during the treatment.

On the completion of the treatment the pieces were drained, washed in Wilson-Kern extractors (Wilson & Kern, 1921) with 9 l. distilled water for 20 hr. and air dried.

Analysis of products

Total N, *amide* N, *amino* N *and arginine.* The methods have been described previously (Bowes & Kenten, 1948*a*, *b*).

Lysine. This was determined using a specific decarboxylase (Gale, 1945). Though this method may also determine an unknown fraction of the hydroxylysine it gives an indication of the extent to which ϵ-amino groups are modified. Recent evidence (Heathcote, 1948) suggests that the apparent reaction of hydroxylysine with lysine decarboxylase may in fact be due to contamination of the hydroxylysine with lysine.

O-*Methyl groups.* These were determined as methyl iodide using a semimicroalkoxyl apparatus (Clark, 1932, 1939; Viebock & Schwappach, 1930). Methionine also yields methyl iodide by this method (Baernstein, 1932; 1936): the oxhide and sheepskin collagens gave values of 0·08 and 0·05% of methyl, respectively.

N-*Methyl groups.* The method is described by Pregl (1930). *O*-Methyl and *N*-methyl contents are expressed as a percentage of methyl on moisture- and ash-free collagen and are corrected for the methionine content of the collagen.

Chloride and sulphate. The former was determined as described by Highberger & Moore (1929). In the determination of $SO_4^=$, methylated collagen (3 g.) was heated for 3 hr. on a steam bath with 30 ml. 2N-HCl. The solution was evaporated to dryness, the residue dissolved in 100 ml. hot water and boiled with charcoal (Norite) for 15 min. The solution was filtered, the charcoal extracted with boiling water, and the combined filtrates neutralized with 2 ml. 5N-HCl, and the $SO_4^=$ determined as $BaSO_4$. Collagen gave no $SO_4^=$ by this method, and recovery tests showed an accuracy of ±5%.

Tannin in combination with collagen. This was determined by drying in a vacuum oven at 100–102° for 6 hr. and weighing. The amount of tannin fixed/100 g. moisture- and ash-free collagen was then calculated from the increase in weight of the collagen.

Chromium. The method used is described by Davies & Innes (1944). Results are expressed as g. Cr_2O_3/100 ml. and mmol. Cr/g. moisture- and ash-free collagen.

Formaldehyde. The treated collagen (2 g.) was steam-distilled with 40 ml. 2N-H_2SO_4, 500 ml. distillate being collected. The formaldehyde was precipitated from suitable samples of distillate with dimedone (Yoe & Reid, 1941).

Shrinkage temperature. The temperature at which shrinkage occurs in water is a property characteristic of both collagen and treated collagens. It has been related to cross linking in the protein structure (Theis, 1946). A modified form of the apparatus described by Küntzel (1943) was used, in which a stream of air was blown continuously round the side arm to aid circulation. When the shrinkage temperature exceeded 100° the tanned collagens were wetted by immersion in water under reduced pressure and the determination then made in liquid paraffin. The shrinkage temperature so determined is the same as that determined in water under pressure.

Titration and swelling curves. These were determined as previously described (Bowes & Kenten, 1948*a*, *b*).

RESULTS

Except where otherwise stated, results are expressed on a moisture- and ash-free basis. Amino-N contents, etc., are expressed as mmol./g. rather than as a percentage of total N, since variations in the N content of the modified collagens render the latter method of expression unsuitable for comparative purposes.

Analysis

Deamination. The deaminated collagens had the characteristic orange-yellow colour associated with proteins which have been treated with nitrous acid (cf. Philpot & Small, 1938). Reaction of the amino groups was almost complete in all the samples examined (Table 1). As with other proteins (Steudel & Schumann, 1929; Wiley & Lewis, 1930) there was some loss of arginine (*c.* 20%).

The amide N was higher than that of the original collagen, and this additional NH_3 may have come from —NH.CN groups formed by the action of the nitrous acid on guanidino groups (see p. 149). The increase in amide N was approximately equal to the decrease in arginine content.

Methylation. The methylated collagens varied from brown to grey in colour and in two cases were almost black. They had a smell of methylamines. The methoxyl content of the different collagens treated with methyl sulphate varied from 0·94 to 1·57% (as methyl), the maximum methoxyl content being attained in 14 methylations (see oxhide collagen, Table 2). Variations in methoxyl content are probably due to slow hydrolysis of these groups during washing (see below). Methylation with methyl bromide introduced rather fewer methoxyl groups. Deamination of the collagen did not affect the extent of methylation with methyl sulphate, but increased the methoxyl groups introduced by methyl bromide to the same value as that obtained with methyl sulphate. This suggests that the lower values obtained with methyl bromide on untreated collagen are due, not to inability to introduce the same number of methoxyl groups as methyl sulphate, but to the difficulty of keeping the collagen at a suitable pH for the reaction to proceed to completion; diminution in the number of basic groups will reduce the change in pH of the collagen which occurs as a result of methylation of the carboxyl groups, and hence makes it easier to keep the collagen at the required pH.

Methylated collagen fixes $SO_4^=$ and Cl^- approximately equivalent to the methoxyl groups introduced. Blackburn *et al.* (1941) found that when wool was methylated with

Table 1. *Analyses of collagens and deaminated collagens*

(Results expressed on moisture- and ash-free basis except where otherwise stated.)

	Untreated	Treated with HNO_2		Untreated	Treated with HNO_2
	C	DC_1	DC_2	*A*	DA_1
Moisture (%) air dry	16·2	6·3*	16·0	18·1	14·1
Ash (%) air dry	0·23	1·78	0·23	0·14	0·58
Total N (%)	17·2	16·9	—	17·3	17·3
Amide N (mmol./g.)	0·25	0·38	0·39	0·30	0·38
Amino N (mmol./g.)	0·36	0·05	—	—	0·04
Lysine† (mmol./g.)	0·30	—	Nil	0·31	0·01
Arginine (mmol./g.)	0·44	—	0·35	0·43	0·37

* The low value is due to the collagen having been dried in a desiccator over anhydrous $CaCl_2$ for some days; other samples air-dry.

† May include some hydroxylysine.

Table 2. *The methylation of collagen and deaminated collagen with methyl sulphate and with methyl bromide*

Sample	OCH_3 groups introduced (as CH_3)		Anions (m-equiv./g. collagen)		
	(%)	(mmol./g.)	Chloride*	Sulphate	Total
Methylated with methyl sulphate					
Oxhide collagen (free carboxyl groups, 0·87 mmol./g.):					
Methylated 14 times MN_1	1·19	0·79	Nil	0·64	0·64
Methylated 14 times MN_2	0·94	0·63	Nil	0·52	0·52
Methylated 16 times MN_3	1·37	0·91	Nil	0·78	0·78
Methylated 16 times MN_4	1·22	0·81	Nil	0·70	0·70
Methylated 18 times MN_5	1·15	0·77	Nil	0·65	0·65
Methylated 18 times MN_6	1·24	0·83	Nil	0·75	0·75
Collagen *A* (free carboxyl groups 0·96 mmol./g.):					
MA_1	1·25	0·83	0·61	—	—
MA_2	1·32	0·88	0·73	0·07	0·80
MA_3	1·49	0·99	0·58	0·40	0·98
MA_4	1·32	0·88	0·50	0·34	0·84
Collagen *C* (free carboxyl groups, 1·01 mmol./g.):					
MC_1	1·32	0·88	—	—	—
MC_2	1·57	1·05	0·52	0·52	1·04
MC_3	1·18	0·79	0·65	—	—
MC_4	1·04	0·69	—	—	—
Collagen *A* after treatment with nitrous acid:					
MDA_1	1·32	0·88	0·42	0·05	0·47
Methylated with methyl bromide					
Collagen *C*:					
Methylated 14 days MC_5	0·35	0·23	0·11	—	0·11
Methylated 28 days MC_6	0·85	0·57	0·48	—	0·48
Collagen *C* after treatment with nitrous acid:					
Methylated 28 days MDC_1	1·22	0·82	0·30	—	0·30

* Arises from displacement of $SO_4^=$ by Cl^- during immersion of methylated collagen in NaCl.

methyl iodide and bromide, I^- and Br^- were held in excess of the methoxyl groups, and attributed this to *N*-methylation. Attempts to determine the percentage of *N*-methyl groups were unsuccessful. Under the conditions of Pregl's method proteins and amino-acids yield appreciable amounts of volatile iodides, and large and variable values for the apparent *N*-methyl are obtained (Burns, 1914; Geake & Nierenstein, 1914; Lindley & Phillips, 1947). Determination of *N*-methyl groups in methylated collagen gave values of the same order as those obtained with the original collagen. Though it may be unwise to conclude that no *N*-methylation takes place, it appears that it only occurs to a small extent, if at all. The amino N of the collagen methylated with methyl bromide (MC_5) and with methyl sulphate (MC_4) was 0·24 and 0·31 mmol./g., respectively, compared with a value of 0·36 mmol./g. for the original collagen. The amino N of collagen MA_4 methylated with methyl sulphate was 0·30 mmol./g.

Stability. Methoxyl groups were only slowly split by hydrolysis when the methylated collagens were immersed in buffer solutions between pH 2 and 8, but outside this range the stability decreased rapidly with rise or fall in pH (see Fig. 1). A similar stability curve for methylated wool constructed from the data of Blackburn *et al.* (1941) is given for comparison. Soaking in water at pH 5·0 for 20 days

reduced the methoxyl content from 1·38 to 1·12 % (as methyl).

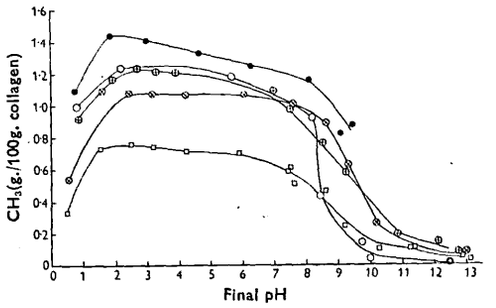

Fig. 1. Stability of OCH_3 groups introduced into collagen, deaminated collagen and wool in pH range 0·5–13·0. Percentage CH_3 after soaking in universal buffer solution for 2·5 days. ⊙—⊙, collagen esterified with methyl sulphate; ⊡—⊡, collagen esterified with methyl bromide; ⊕—⊕, collagen deaminated and esterified with methyl sulphate; ⊗—⊗, collagen deaminated and esterified with methyl bromide; •—•, wool esterified with methyl sulphate (from Blackburn *et al.* 1941).

Table 3. *Soluble nitrogen liberated from methylated collagen after 3 days' exposure to alkaline solutions*

Sample	pH of solution	Soluble N (mg./g.)	OCH_3 groups removed (g. CH_3/100 g. collagen)
	Untreated		
C	10·10	2·4	—
C	12·08	3·0	—
C	12·27	3·4	—
C	12·32	3·7	—
C	12·69	4·8	—
C	13·00	6·6	—
A	12·08	3·2	—
	After methylation with dimethyl sulphate		
MC_4	7·12	2·6	0·28
MC_4	7·42	4·9	0·29
MC_4	8·60	4·9	0·33
MC_4	8·79	10·4	0·59
MC_4	10·73	15·9	0·98
MC_4	11·80	21·5	1·01
MA_2	12·09	4·9	—
MA_3	12·12	6·3	—
MA_4	12·10	8·3	—
MDA	12·16	5·7	—
	After methylation with methyl bromide		
MC_5	8·76	1·1	0·32
MC_5	9·28	1·1	0·27
MC_5	10·20	1·1	0·33
MC_5	11·61	1·6	0·20
MC_5	12·01	1·5	0·34
MC_5	12·24	2·1	0·33

At high pH values loss of methoxyl groups from collagen methylated with methyl sulphate is accompanied by the production of an appreciable amount of nitrogen in the solution (see Table 3). Collagen methylated with methyl bromide, on the other hand, gave less soluble nitrogen than the untreated collagen. The solutions smelt of methylamines and gave a positive test for dimethylamine (Dowden, 1938). This smell was also noted by Blackburn *et al.* (1941) during the methylation of wool.

It was considered possible that the N in solution was related to the methylation of one of the basic groups of the collagen and specific degradation of this group on treatment in alkaline solution. The arginine content of the collagen as determined by Vickery's method, however, was unchanged by methylation and treatment with alkali, suggesting that the soluble N was not derived from guanidino groups. (There is some evidence, however, that some methylation of guanidino groups does take place. Lindley & Phillips (1947) found no decrease in the arginine content of methylated wool as determined by Vickery's method, but tests on the flavaniate precipitate indicated that some change had occurred.) The solution gave only a slight positive reaction for protein, and the results as a whole suggest that the soluble nitrogen is derived from general hydrolysis of the collagen to polypeptides and amino-acids.

Titration curves

Deaminated collagen. The titration curve of deaminated collagen (DC_2) is given in Fig. 2. Deamination has caused a shift in the isoelectric point from 5·5 to 4·5, a decrease in the acid-binding capacity, an increase in the base-binding capacity immediately on the alkaline side of the isoelectric point, and a slight increase in the total base bound at pH 12·5. The decrease in acid binding can be attributed mainly to loss of amino groups, but consideration of the free amino N of the original collagen (0·38 mmol./g.) shows that at least 0·07 mmol./g. of some other basic group has also been affected. The increase in base-binding from the isoelectric point to pH 7·0 corresponds to the decrease in acid-binding capacity, and may be attributed to titration of carboxyl groups which have reverted to the uncharged form on the removal of the basic groups. The increase in base-binding capacity at pH 12·5 indicates that about 0·18 mmol./g. of some group or groups not titrating in the original collagen is now binding base between pH 7 and 12·5.

Methylated collagen. The titration curves of collagens MC_2 and MC_4 (methylated with dimethyl sulphate) and MC_5 (methylated with methyl bromide) are shown in Fig. 2. In agreement with the hypothesis that methylation involves esterification of ionized carboxyl groups which are the hydrogen ion-fixing groups in the original protein, whilst the basic groups remain charged, the acid-binding capacities of the methylated collagen are decreased by an amount approximately corresponding to their methoxyl contents. The decrease in acid-binding capacity, however, is slightly less than the methoxyl content of the collagen, possibly owing to

loss of methyl groups by hydrolysis during contact with the solution. Also, since the isoelectric point of the methylated collagen is about one pH unit higher than that of the original collagen, some of the imidazole groups of histidine which were previously charged, and, therefore, titrated with alkali, will have lost their charge and now contribute to the acid-binding capacity.

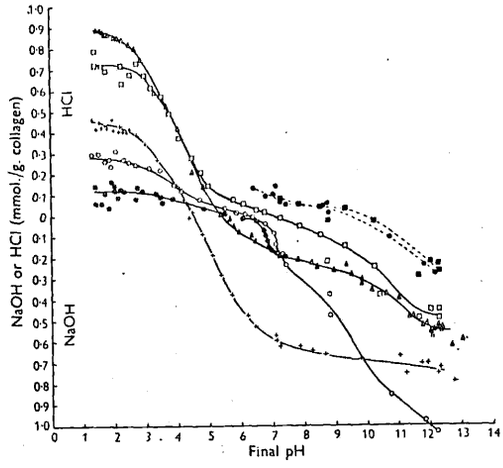

Fig. 2. Titration curves of collagen and modified collagens. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate, CH_3 content 1·04%; •—•, esterified with methyl sulphate, CH_3 content 1·04%; corrected for hydrolysis of OCH_3 groups; ⊗—⊗, esterified with methyl sulphate, CH_3 content 1·57%; ⊡—⊡, esterified with methyl bromide, CH_3 content 0·35%; ■—■, esterified with methyl bromide, corrected for OCH_3 hydrolysis; ⊕—⊕, deaminated and esterified with methyl sulphate, CH_3 content 1·29%.

The alkaline section of the curve is more difficult to interpret for, except over a comparatively small pH range, hydrolysis of methoxyl groups takes place with the consumption of alkali. The methoxyl content of the collagens, after contact with the solution, was determined and a correction applied for the alkali consumed; this is only approximate, but the corrected results suggest that appreciably less alkali is bound by the methylated than by the original collagen. In the case of collagen methylated with methyl bromide, the titration curve shows that less alkali is bound even before the correction is applied.

Swelling curves

Deaminated collagen. The uptake of water by the deaminated collagen (see Fig. 3) differs from that of the original collagen in a manner broadly corresponding to the differences in the titration curves (Fig. 2), but at all pH values the water uptake is rather greater than would be expected from consideration of the changes in the reactive groups. This suggests that deamination has decreased the cohesion of the collagen so allowing it to take up more water.

Methylated collagen. In the pH range of stability of the methoxyl groups, i.e. 2–9, the water uptake of methylated collagen is constant, and, when the collagen is fully methylated, is approximately the same as the maximum water uptake of the untreated collagen at pH 2·0 (see Fig. 3). This high water uptake over such a wide pH range may be ascribed to the presence of anions in electrovalent association with the basic groups, and consequent setting up of a Donnan membrane equilibrium and a swelling pressure (Donnan, 1911, 1924; Bolam, 1932). In this respect, methylated collagen corresponds to collagen at the point of maximum combination with acid.

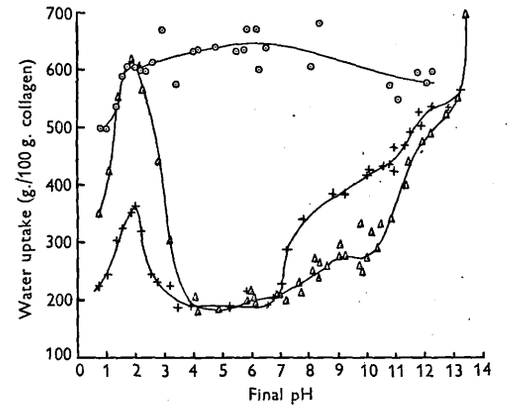

Fig. 3. The uptake of water by untreated △—△, deaminated +—+, and methylated collagen ⊙—⊙, at pH values from 0·5 to 13·5.

Combination of untreated and modified collagens with tannic acid, mimosa tannins, chromium and formaldehyde

Tannins. The conditions of treatment were the same for all the modified collagens, and it is reasonable, therefore, to assume that any differences in the amount of tannin bound are primarily due to modification of the collagen.

Modification of the amino and carboxyl groups of the collagen affects the combination of both tannic acid (hydrolyzable tannin) and mimosa (condensed tannin) with collagen in a similar manner (Figs. 4–6). Deamination decreased the amount of tannin bound at pH values below 4·0, but had little effect at higher pH values, and esterification of the carboxyl groups caused maximum combination of tannin to take place at much higher pH values, and to remain constant at this maximum over a wide pH range; this was especially marked with mimosa tannin (Fig. 6).

The addition of 0·5 M-NaCl decreased the amount of tannic acid bound by untreated and deaminated

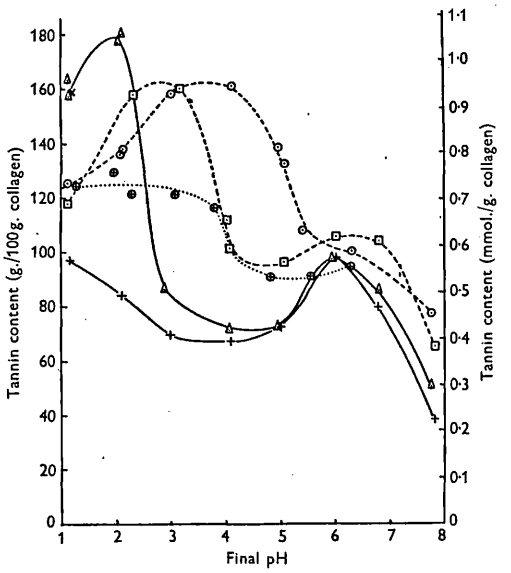

Fig. 4. Combination of tannic acid with collagen and modified collagen in the absence of added NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide; ⊕—⊕, deaminated and esterified with methyl sulphate.

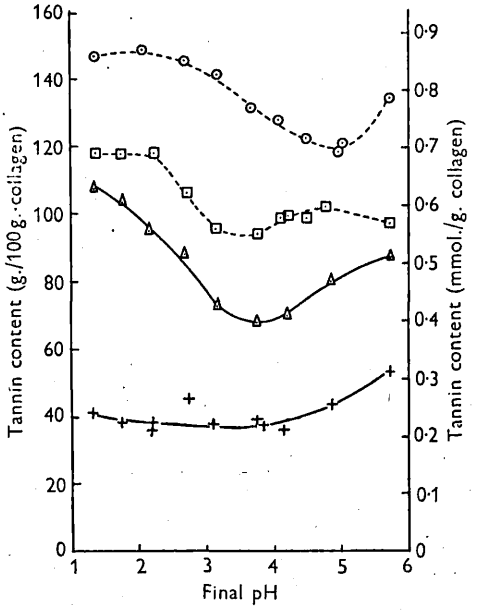

Fig. 5. Combination of tannic acid with collagen and modified collagen in the presence of 0·5 M-NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide.

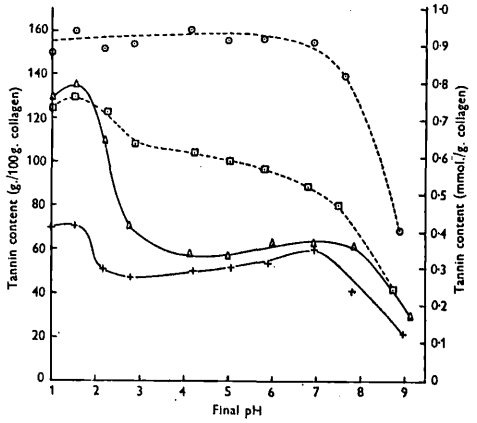

Fig. 6. Combination of mimosa tannins with collagen and modified collagen in the absence of added NaCl. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊡—⊡, esterified with methyl bromide.

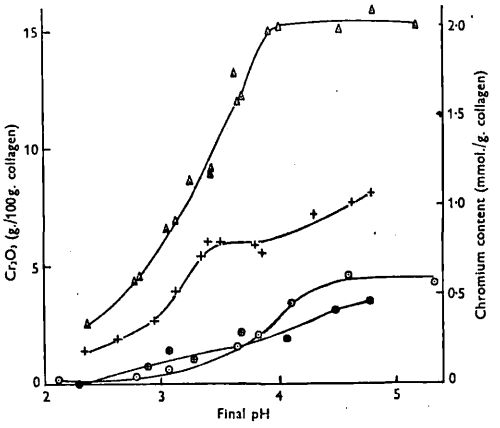

Fig. 7. Combination of chromium with collagen and modified collagen. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate; ⊕—⊕, deaminated and esterified with methyl sulphate.

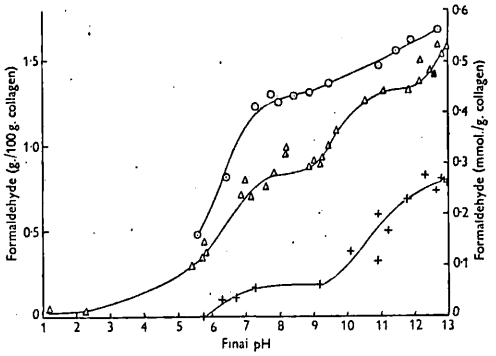

Fig. 8. Combination of formaldehyde with collagen and modified collagen. △—△, untreated; +—+, deaminated; ⊙—⊙, esterified with methyl sulphate.

collagen, and by collagen methylated with methyl bromide, especially at low pH values. The tannic acid bound by the collagen methylated with methyl sulphate was only slightly decreased at pH values below 5·0, and at higher pH values was appreciably increased by the presence of NaCl.

The shrinkage temperatures of the collagen and modified collagen were increased from about 60 to between 66 and 84° by combination with tannic acid or mimosa tannin, maximum values in general being obtained when the pH of the tannin solution was between 3·0 and 4·0. The increase in shrinkage temperature was rather greater with mimosa tannin than with tannic acid, possibly because of the lower acidity of mimosa tannin (Hobbs, 1940). The deaminated and methylated collagens tended to have lower shrinkage temperatures than the original collagen treated under the same conditions, and variations of shrinkage temperature with the pH of the tannin solutions were less.

Chromium. The chromium bound by the original collagen increased sharply with increase in pH of the chromium sulphate solution up to 4·0, and then remained constant over the pH range studied (Fig. 7). Deamination decreased the combination of chromium over the whole pH range and reduced the maximum amounts combined by approximately half. Methylation decreased combination to a much greater extent. Chromium hydroxide was precipitated on the surface of the methylated collagens at the higher pH values and was difficult to remove; this, coupled with the possibility that some groups are displaced by chromium during the treatment, makes it not unreasonable to assume that in the absence of carboxyl groups no chromium would be bound by collagen. Deamination of the methylated collagen caused only a further small decrease in the chromium bound. Although the amount of chromium bound by the deaminated collagen was less than that bound by the original collagen, the maximum shrinkage temperature obtained was the same. Treatment with chromium sulphate caused no change in the shrinkage temperature of methylated colagen.

Formaldehyde. At pH values below 2·0, negligible amounts of formaldehyde were bound by both the untreated and modified collagens: from pH 2 to 8 the amount bound increased, remained constant between pH 8 and 9, and then increased again at higher pH values (see Fig. 8). Deamination greatly reduced combination between pH 2 and 9. The amount of formaldehyde bound by the untreated collagen up to pH 9·0, and the decrease in combination following deamination, correspond to considerably less than the combination of one formaldehyde molecule by each amino group; similarly, the amount bound from pH 9·0 to 13·0, the upper limit of pH covered, only corresponds to the combination of one molecule of formaldehyde by each of half the total number of guanidino groups.

With untreated and methylated collagen the combination of as little as 0·4 g. formaldehyde/100 g. raised the shrinkage temperature to its maximum value (78–80°), whereas the combination of 0·8 g. formaldehyde/100 g. left the shrinkage temparature of the deaminated collagen unchanged.

DISCUSSION

Deamination. Under the experimental conditions employed, treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by about 20 %. The course of the reaction with the amino groups is well known, but little attention appears to have been given to the reaction with the guanidino groups. Bancroft & Belden (1931) and Bancroft & Ridgway (1931) have shown that guanidine reacts with nitrous acid to give cyanamide and ammonia, and it is possible that a similar reaction takes place with the guanidino groups in proteins, namely,

$$—CH_2,NH.C(=NH)(NH_2) + HONO \rightarrow —CH_2.NH.CN + NH_3.$$

In agreement with this hypothesis, Kanagy & Harris (1935) have shown that ammonia is formed when arginine is treated with nitrous acid; this ammonia will react with nitrous acid to give the excess nitrogen observed in the Van Slyke determination of amino N (Plimmer, 1924; Hunter, 1929; Kanagy & Harris, 1935; Lieben & Loo, 1942; Van Slyke, Hiller & Dillon, 1942). This hypothesis would also explain the higher amide N of the deaminated collagen compared with the original collagen since, under the conditions of the determination, the —NH.CN group would probably yield ammonia. This increase in amide N is of the same order as the decrease in the arginine content of the collagen.

The reaction postulated above is also consistent with those changes in the titration curve which cannot be attributed to loss of amino N. Loss of the strongly basic guanidino groups accounts for the decrease in acid-binding capacity; carboxyl groups equivalent to the guanidino groups lost will revert from the zwitterion state (—COO^-) to the uncharged state, and will titrate between the isoelectric point and pH 7·0, and the —NH.CN groups, being feebly acidic, will titrate between pH 7 and 12, thus accounting for the increased base-binding capacities in these ranges. Since guanidino groups do not titrate with base up to pH 12·5 (Bowes & Kenten, 1948*a*), the total base-binding capacity should be increased by an amount equal to twice the —NH.CN groups formed, i.e. base bound by the additional

un-ionized carboxyl groups plus that bound by —NH.CN groups. On this basis, and assuming that 0·05 mmol./g. amino groups remain (see Table 1), the curve indicates that about 0·10 mmol./g. guanidino groups are converted to cyanide groups. (Decrease in acid binding in excess of that due to amino groups, 0·12 mmol./g.; base bound between 7 and 12, 0·10 mmol./g., and total increase in base-binding capacity, 0·20 mmol./g.) This figure is in good agreement with decrease in arginine content found by analysis, 0·09 mmol./g.

Esterification. Like the carboxyl groups of amino-acids, those of wool, silk, gelatin, and collagen can be esterified with methyl sulphate and methyl bromide (Blackburn *et al.* 1941; Blackburn & Phillips, 1944).

The evidence obtained in the present investigation confirms this view with respect to collagen. The reaction would appear to necessitate the presence of basic groups equivalent to carboxyl groups; it was found, however, that deamination did not decrease the extent of methylation. Blackburn & Phillips (1944) considered that the lysine content of wool is so low that its removal does not materially affect the number of basic groups. This explanation is not applicable to collagen since amino groups form about one third of the total basic groups, and a more likely explanation is that as ionized carboxyl groups are esterified, further carboxyl groups ionize under the influence of the guanidino groups, so that eventually all carboxyl groups are esterified.

Consideration of the values for the free carboxyl groups in the untreated collagens, as indicated by analysis and by their titration curves (Bowes & Kenten, 1948*b*), shows that all the methoxyl groups introduced can be accounted for on the basis of esterification of carboxyl groups. In three experiments, the number of methoxyl groups was slightly in excess of the number of free carboxyl groups but this excess was within the experimental error. Blackburn *et al.* (1941) and Blackburn & Phillips (1944) found that the methoxyl groups introduced by methyl sulphate exceeded the number of free carboxyl groups believed to be present in wool, silk, gelatin, and collagen, and suggested that methylation also occurred at certain 'activated' peptide links. It is now known (Bowes & Kenten, 1948*a*) that the values taken for the number of carboxyl groups in gelatin and collagen were low, and the present work shows that with collagen there is no necessity to postulate any form of *O*-methylation other than that of carboxyl groups. It is possible that future determinations of the dicarboxylic acids in wool and silk will obviate the necessity of assuming that these proteins undergo peptide methylation.

The low amino-nitrogen values of the methylated collagens suggest that some *N*-methylation has occurred, especially with methyl bromide. The lower base-binding capacity of the methylated collagens as compared with that of untreated collagen may also be due to *N*-methylation, for although the introduction of a methyl group will not eliminate the basic characteristics of the amino group, it will, by analogy with the methylamines, increase the pK of these groups and hence cause a shift in the titration curve to higher pH values. It is possible that the titration curves of the methylated collagen would show the same maximum base-binding capacity as the original collagen if carried to higher pH values. It is also possible that the reduction in base-binding capacity of the collagen methylated with methyl sulphate is related to the presence of nitrogen in the solutions after contact with the collagen; if this nitrogen were present as a base (for instance, as methylamine) it would titrate and so reduce the apparent fixation of base by the collagen.

Combination of collagen and modified collagens with tannins, chromium and formaldehyde. Although tannic acid, in contrast to the tannins of mimosa, contains acidic groups (Sunthankar & Jatker, 1938; Abichandani & Jatker, 1938; Cheshire, Brown & Holmes, 1941) which might be expected to form salts with the basic groups of the collagen, the mode of combination with collagen appears to be the same with both materials. The results as a whole are consistent with the supposition that combination of tannin is related to the positive charge carried by the protein. With the untreated collagen, combination is greatest between pH 1·5 and 2·0 when the collagen carries its maximum net positive charge, decreases as the pH increases and the net positive charge decreases, and eventually is reduced to negligible proportions at pH 8·0–9·0 when the basic groups begin to lose their positive charge and the protein carries a net negative charge. Deamination which decreases the positive charge on the collagen, decreases combination of tannin, and esterification, which causes the collagen to carry its maximum positive charge over the whole pH stability range of the methoxyl groups, causes combination of tannin also to be at a maximum over this range. Experiments indicate that the molecular weight of tannic acid and mimosa tannin is 1700 (Brintzinger & Brintzinger, 1931; Humphreys & Douglas, 1937), and the equivalent weight of tannic acid is of the same order (Cheshire *et al.* 1941). Using these figures, an interesting comparison can be made between the amounts of tannin bound and the acid-binding capacity of the collagens (see Table 4). The molecular amounts of tannin combined with the untreated and deaminated collagens between pH 1·5 and 2·0 are of the same order as their acid-binding capacities; deamination halves the acid-binding capacity and also halves the amount of tannic acid and mimosa tannin bound. This suggests that one molecule of tannin is associated with each basic

group. Although the amounts of tannin bound are dependent on the concentration of tannin in the solution, the time of treatment and method of washing, it would seem reasonable to assume that there is some significance in these stoicheiometric relationships.

Table 4. *Acid and tannin bound by collagen and deaminated collagen*

(Results in mmol./g.)

Collagen	Acid-binding capacity	Tannin bound between pH 1·5 and 2·0	
		Tannic acid	Mimosa tannin
Untreated collagen *A*	0·88	1·0	0·80
Deaminated collagen DA_1	0·44	0·50–0·55	0·41

The present results emphasize the essential part played by the carboxyl groups in the binding of chromium, and strengthen the current view that the fixation of metals involves complex formation with the carboxyl groups of the protein in a manner analogous to the complex formation which occurs with the carboxyl groups of organic acids (for review of literature see Bowes, 1948). The amino groups also appear to be involved since deamination decreased the amounts bound by the untreated collagen, though not by the methylated collagen. These findings are consistent with the hypothesis that combination of chromium involves co-ordination of both carboxyl and amino (or other basic groups) with the same chromium complex. A similar hypothesis has been put forward to account for the fixation of calcium by proteins (Greenberg, 1944), and for the high thermal stability of chrome-tanned leather (Küntzel & Riess, 1936).

Although the validity of stoicheiometric relationships between the amounts of chromium bound and the reactive groups of the collagen may, in view of the complexity of the system, be open to question, it is of interest to note that the amount of chromium bound between pH 4 and 5 approximately corresponds to the fixation of two chromium atoms by each carboxyl group, and the decrease in combination caused by treatment with nitrous acid corresponds approximately to two chromium atoms for each basic group lost. It is probable that the chromium aggregates in the tanning solutions used contained, on the average, two chromium atoms (Bowes, 1948).

The evidence suggests that, in collagen, the amino and guanidino groups are the main centres involved in the binding of formaldehyde, and there is no evidence that the amide groups are concerned under the conditions employed. The importance of the amino groups raising the shrinkage temperature (Gustavson, 1943) is confirmed. Free amino groups also play an essential part in the hardening of casein with formaldehyde (Nitschmann & Hadorn, 1944; Nitschmann & Lauener, 1946), and it is probable that a similar mechanism is involved, namely, the formation of cross links between adjacent polypeptide chains. Nitschmann & Hadorn (1944) and Nitschmann & Lauener (1946) discuss the various ways in which such cross links may be formed and consider that a cross link between ϵ-NH_2 groups and —NH— groups of the peptide link is the most probable. (For further discussion on this point see Fraenkel-Conrat, Cooper & Olcott, 1945; French & Edsall, 1945; Bowes, 1948.)

SUMMARY

1. Collagen has been deaminated with nitrous acid and esterified with methyl sulphate and methyl bromide, and the effect of these treatments on the reactivity of collagen towards acids, bases, tannins, chromium and formaldehyde has been determined. Modification of the guanidino groups by treatment with hypochlorite (Sakaguchi (1925) reaction) was only partially successful; only 40–50% of the arginine was destroyed and there was extensive general breakdown of the collagen.

2. Treatment with nitrous acid removed almost all the amino groups from collagen and decreased the arginine content by approximately 20%. Evidence deduced from analysis and titration curves suggests that the guanidino groups are converted into cyanamide groups.

3. All the *O*-methyl groups introduced into collagen by methyl sulphate and methyl bromide can be accounted for on the basis of esterification of carboxyl groups. There is an indication that some *N*-methylation occurs, especially with methyl bromide.

4. From a study of the combination of tannins, chromium and formaldehyde with untreated, deaminated and methylated collagen, it is suggested that combination of tannins is related to the positive charge carried by the collagen, combination of chromium involves co-ordination of both amino and carboxyl groups of the collagen with the same chromium complex, and combination with formaldehyde occurs mainly with the amino and guanidino groups. Increase in thermal stability results only from combination of formaldehyde with amino groups.

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THE EFFECT OF MODIFICATION OF THE REACTIVE GROUPS OF COLLAGEN ON THE FIXATION OF TANNING AGENTS.

By J. H. Bowes and R. H. Kenten.

It is generally agreed that the reactive groups of collagen are involved in the fixation of tanning agents, and modification of these groups and determination of its effect on the fixation of the different types of tan is one method of gaining information regarding the mechanism of tanning.

Thomas and co-workers[1, 2] were probably the first to undertake an investigation on these lines. They treated collagen with nitrous acid to modify the amino groups, and determined its effect on the fixation of vegetable tannins[1] and chromium[2]. Unfortunately they did not determine the extent to which the amino groups were removed, or other groups affected, but only the nitrogen lost during the treatment, and their results, therefore, are qualitative rather than quantitative. With both vegetable tannins and chromium, treatment with nitrous acid caused a marked decrease in fixation, and it would appear from their results that the amino groups are involved in the fixation of these tans, although the possibility that the decrease is due to the change in the electrical condition of the collagen is not excluded. Their results have subsequently been confirmed by Gustavson[3] with chromium, using anionic chromium complexes ; by Page and Holland[4], Chang, Yen and Chen[5], and Lollar[6] with vegetable tannins. Numerous workers[7-12] have determined the effect of deamination on the fixation of formaldehyde ; all report a marked decrease, and it is generally agreed that reaction with the amino groups is an essential factor in formaldehyde tanning.

Apart from the work of Highberger and Salcedo[13] and of Gustavson[10] on the effect of modification of the guanidino group on the fixation of formaldehyde, no work has been done on the effect of modification of the other reactive groups, guanidino, carboxyl, amide, and hydroxyl, which, especially the guanidino and carboxyl groups, are likely to play an important part in the fixation of tans.

The present work is a study of the fixation of vegetable tannins, chromium, and formaldehyde by collagen which had been treated with nitrous acid, alkaline hypochlorite, or dimethyl sulphate or methyl bromide with the object of modifying the amino, guanidino and carboxyl groups respectively. A study of the effect of modification of these three groups concurrently using the same initial raw material is likely to give more information regarding the mechanism of tanning than the study of the tanning properties of a collagen of which only one particular group has been modified.

Preparation of Collagen and Modified Collagens.

Collagen.

Collagen was prepared from commercially limed and pickled cape sheepskins. The skins were depickled, and acetone dehydrated. The edges of the skin were discarded, the remainder cut into small strips, degreased with light petroleum, washed repeatedly in distilled water and acetone dehydrated.

TABLE I.

ANALYSES OF COLLAGEN AND MODIFIED COLLAGENS.

Collagens C, DA_1, HC_1, MA_1, MC_5, and DMA were used for experiments with tannic acid in the absence of added salt.

Collagens C, DC_1, HC_1, MC_3, and MC_5 were used for experiments with tannin acid in the presence of salt.

Collagens C, DA_1, HC_2, MA_4, and MC_5 were used for experiments with mimosa.

Collagens B, DA_1, HC_2, MA_2, and DMA were used for experiments with chromium.

Collagens B, DA_1, and MA_5 were used for experiments with formaldehyde.

Collagen	Ash g./100 g air-dry collagen	Total N, g./100 g moisture and ash-free collagen	Millimoles per g. Amino N	Amide N	Arginine	Free carboxyl	O – Me groups	Total basic groups
Untreated collagens.								
Limed collagen A	0·25	17·3	—	0·30	0·43	0·96*	—	0·89
B	0·35	17·1	0·42	0·30	0·42	1·00*	—	0·97
C	0·14	17·2	0·36	0·25	0·44	1·01*	—	0·88
Deaminated collagens.								
DA_1	0·58	17·3	0·04	—	0·37	0·96†	—	—
DC_1	0·24	16·9	0·05	—	0·35	1·01†	—	0·43
Hypochlorite-treated collagens.								
HC_1	0·25	—	0·32	—	0·24	—	—	0·78
HC_2	0·10	—	—	—	0·33	—	—	—
Methylated collagens.								
MA_1	0·10	—	—	— ‡	— ‡	0·13†	0·83	—
MA_2	0·58	16·3	0·31	0·28	—	0·08†	0·88	—
MA_4	—	—	0·30	0·29	0·42	0·08†	0·88	—
MA_5	0·07	—	—	—	—	0·00†	1·05	—
MC_3	0·29	16·7	—	—	—	0·22†	0·79	—
MC_5	0·22	17·2	0·24	—	—	0·78†	0·23	—
DMA	0·23	16·6	—	—	—	0·08†	0·88	—

* Deduced from titration curves (see papers by Kenten and Bowes[14, 15])

† Deduced on the assumption that the number of carboxyl groups are unaffected by the treatment, and in the case of the methylated collagens deducting the methyl content.

‡ It may be inferred that the amide nitrogen and arginine contents of the methylated collagens are the same as those of the original collagens.

DEAMINATED AND METHYLATED COLLAGENS.

Full details of the preparation of the deaminated and methylated collagens, and the steps taken to determine the extent to which the different groups were affected are given elsewhere[15]. Relevant data regarding their composition and the experiment for which each was used is recorded in Table I.

HYPOCHLORITE-TREATED COLLAGEN.

200 g. of collagen (Batch C) in the form of strips 1 cm. × 5 cm. was placed in 2,000 ml. of 1.3% sodium hypochlorite, and the vessel immersed in iced water. When the temperature had dropped to 2°C, 1,500 ml. *N* sodium hydroxide was added dropwise over a period of 15 min., and then a further 2,000 ml. of 5% sodium hypochlorite slowly over a period of 4 hr. The solution was continuously stirred vigorously by a mechanical stirrer. The treatment was continued for 45 min. after the final addition of hypochlorite. The strips were then removed, washed with 0.5 *N* acetic acid for 30 min., and soaked in 0.2 *N* acetic acid for 12 hr. They were finally brought to equilibrium with distilled water and acetone dehydrated.

The treatment caused the collagen to decrease in weight by about 50% while only about half the guanidino group were modified (see Table I). The titration curve of the treated collagen (HC_1) was almost identical with that of the original collagen except for a decrease of 0.1 millimoles per g. in the acid binding capacity. Since alkaline conditions are necessary for the reaction with the guanidino groups to take place, it is probable that the mechanism involved is the same as that postulated by Hellerman and Stock[16], and Warner[17] for the action of alkalis on arginine, namely conversion to ornithine and urea, and that the function of the hypochlorite is to remove the urea formed, and so speed up the reaction. Such a reaction should not affect the acid binding capacity since the guanidino group is replaced by an amino group. The small decrease in acid binding is probably due to loss of ε-amino groups of lysine, or imidozole groups of histidine, both of which are reported to be attacked by alkaline hypochlorite[18]. In view of the considerable loss in weight of the collagen and the possibility of other groups being affected[18], it was considered that the method was not very satisfactory.

Fixation of Vegetable Tannins.

(i) Experimental Method.

Tannic acid and mimosa were chosen as representatives of the hydrolysable and condensed type of tannins because of their ready solubility and their low salt and non-tan content ; tannic acid contains no salt[19] and less than 10% of non-tans (on a soluble solids basis), and mimosa contains little salt[19] and only about 20% of non-tans.

TABLE II.

Addition of Acid or Alkali to Tannin Solutions.

(amounts of acid or alkali added to 100 ml. solution expressed as ml.N. solution)

Tannic Acid—no NaCl		Tannic Acid—0.5M NaCl		Mimosa—no NaCl	
pH	Acid or Alkali added	pH	Acid or Alkali added	pH	Acid or Alkali added
1·1	100 ml HCl	1·3	70 ml HCl	1·5	46 ml HCl
2·0	5 „ „	1·7	40 „ „	2·2	40 „ „
		2·2	0·7 „ „		
3·0	4 „ NaOH	2·7	Nil	2·8	21 „ „
		3·2	5 ml. NaOH		
		3·7	25 „ „		
4·1	25 „ „	4·2	62 „ „	4·2	10 „ „
5·0	70 „ „	4·8	88 „ „	5·0	1·5 „ NaOH
6·0	80 „ „	5·7	100 „ „	6·0	7·5 „ „
6·9	120 „ „			6·9	14 „ „
7·8	200 „ „			7·8	45 „ „
9·0	500 „ „			9·1	200 „ „

2 g. samples of collagen or modified collagen, in the form of pieces about 1 cm. square and a strip 1 cm. x 10 cm. (0.3 g.) for shrinkage temperature, were soaked in water overnight, and then placed in a 100 ml. tan solution containing 10% solid material and adjusted to the required pH. In the experiment using tannic acid in the presence of salt, the pieces were soaked in 0.5M sodium chloride instead of water. The amounts of acid or alkali added in the initial adjustments are given in Table II. No difficulty was found in adjusting the solutions to low pH values, but the addition of large amounts of alkali to tannic

acid solutions containing sodium chloride caused some precipitation. The collagen was left in these solutions for 7 days at laboratory temperature, in which time approximate equilibrium between collagen and solution should have been reached[20]. Every morning and evening the pH was adjusted to the initial value by the addition of hydrochloric acid or sodium hydroxide ; these additions were negligible compared with those made in the initial adjustments.

At the end of 7 days, the collagen was removed and washed in a Wilson-Kern extractor for 24 hr. using 9 litres of distilled water. The pieces were air-dried and then, with the exception of the piece for shrinkage temperature determination, dried in a vacuum oven at 100°C. for 6 hr. The pieces were weighed, and the amount of tan fixed by 100 g. moisture- and ash-free collagen calculated from the increase in weight. In the first experiment the total nitrogen of the tanned pieces was also determined, and the amount of collagen present calculated, using the appropriate factor for the collagen in question.

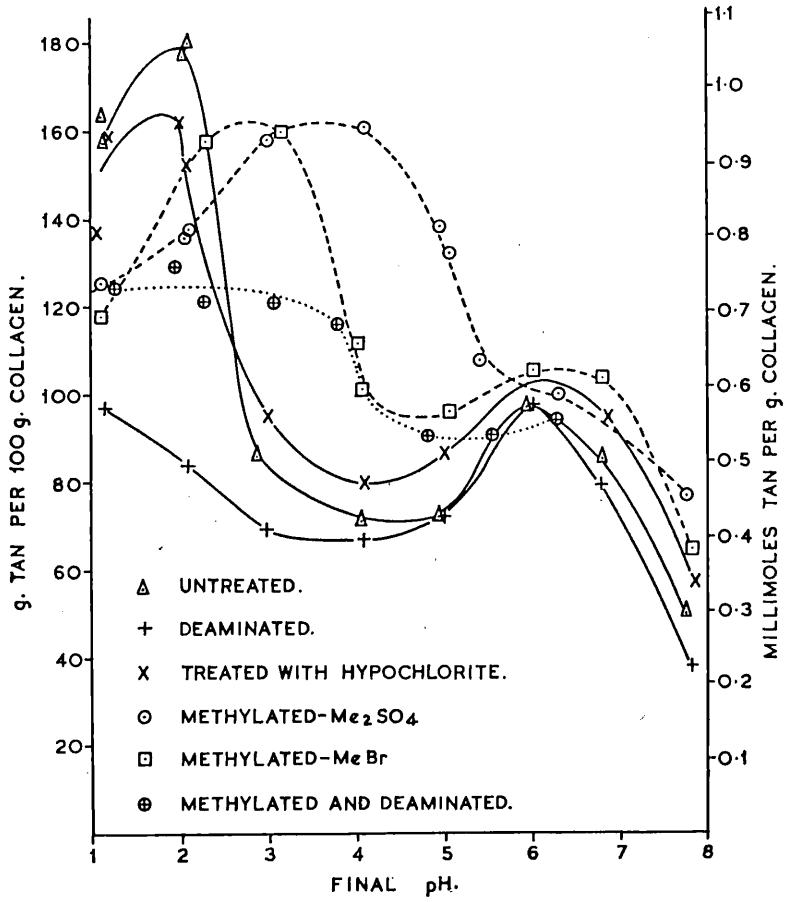

FIG. 1.

The fixation of tannic acid by collagen and modified collagen without the addition of sodium chloride.

The amount of tan fixed was then obtained by subtraction, and the results again expressed as tan fixed per 100 g. collagen. These two methods of calculating the amount of tan fixed were in good agreement, and it was decided that determination of the increase in weight only should be made in the subsequent experiments.

Tests showed that results were reproducible to within $\pm$ 5%.

The tan fixed per 100 g. moisture- and ash-free collagen was plotted against the final pH of the tanning solution (Figs. 1, 2, and 3).

The shrinkage temperatures were determined, using a modified form of the apparatus described by Kuntzel [21], in which a stream of air was continuously blown round the side arm to aid the circulation of the water.

(ii) Experimental Results.

The amount of tan fixed depends on a number of factors besides the condition of the collagen, such as concentration of tan[5, 6, 22-26], time[20, 22], and temperature[27, 28] of tanning. In the present experiments the tanning conditions were designed to give a comparatively high fixation of tan, and the washing procedure to give values approximating to those for Wilson-Kern fixed tans[29]. The conditions were the same for all samples of collagen, and it is, therefore, reasonable to assume that any differences in the amount of tan fixed are due to the treatments given to the collagen. Such differences may arise from alterations in fibre structure leading to increased accessibility of the fibres, as well as from modification of the reactive groups, and this factor must be borne in mind in considering the results. A study of the swelling curves of

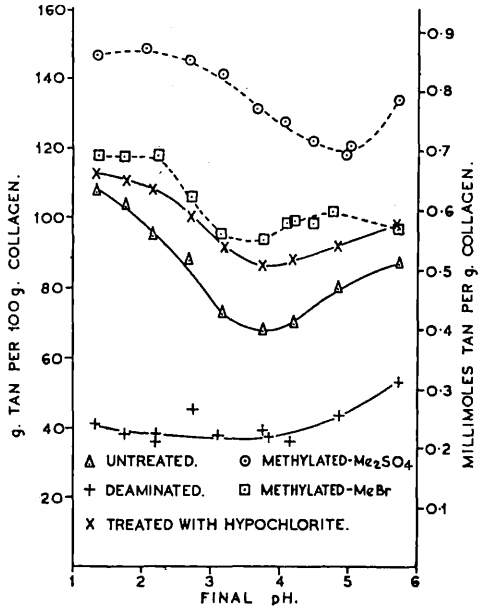

Fig. 2.

The fixation of tannic acid by collagen and modified collagen with addition of 0·5*M* sodium chloride.

the modified collagens[15], however, suggests that the treatments have had little effect in loosening the fibre structure.

Tan may be fixed in the following ways[30] :—

(1) by salt formation.

(2) by formation of hydrogen bonds between the tan molecule and particular groups in the collagen.

(3) by " adsorption."

Tannic acid may be fixed by all three of these mechanisms, whereas,under tanning conditions, mimosa cannot enter into salt formation with any of the reactive groups of collagen and can only be fixed by the last two mechanisms.

(a) *Tannic Acid without the addition of Sodium Chloride.*

The pH-fixation curve for the untreated collagen is similar in shape to that obtained by Thomas and Kelly[24]. Fixation of tan is greatest at about 2.0, it then decreases as the pH rises to 4.5, increases again to a maximum at pH 6.0, and finally decreases rapidly as the pH is further increased.

Deaminated collagen (see Fig. 1) fixes much less tannic acid than the original collagen at low pH values, but about pH 4.0 there is little difference in fixation (*cf* Thomas and Foster[1] who found an increased fixation between pH 4 and 7). Thus, it would appear that the increase in fixation which takes place between pH 4.0 and 2.0 is related to the presence of amino groups. Treatment with hypochlorite and modification of some of the guanidino groups also slightly decreases fixation at pH 2.0 but increases it at pH values above 3.0. As it seems probable that this treatment converts guanidino groups mainly to amino groups[14] it would not be expected to have much effect on fixation. In view of the doubt regarding the exact effect of the hypochlorite treatment it is perhaps, unwise to draw any definite conclusions from this curve.

Esterification of the carboxyl groups causes the maximum fixation to be attained at higher pH values (pH 4.0 with methyl sulphate, and pH 3 with methyl bromide), and the fixation remains constant at this maximum over a wider pH range.

The collagen which had been deaminated and methylated also attains its maximum fixation of tan at a higher pH value than the original collagen, and the fixation remains constant between pH 1 and 4. It is difficult to see why this collagen fixes greater amounts of tan than the collagen which had only been deaminated ; a possible explanation is that the combined effect of the two treatments caused a general loosening of the fibre structure.

There is little variation in the shrinkage temperature of the tanned samples either with change in pH or from one collagen to another. On the whole, the shrinkage temperature tends to vary inversely as the tan content, being highest in the pH range 3 to 4 and at pH 7 to 8. The shrinkage temperature of the deaminated and hypochlorite-treated collagen is rather lower and varies less with pH than that of the original collagen.

TABLE III.

SHRINKAGE TEMPERATURE OF VEGETABLE-TANNED SAMPLES.

Untreated collagen		Deaminated collagen		Hypochlorite-treated collagen		Collagen methylated with CH_3Br		Collagen methylated with $(CH_3)_2SO_4$	
Final pH	S.T., °C.	Final pH	S.T., °C.	Final pH	S.T., °C.	Final pH	S.T., °C.	Final pH	S.T., °C.
TANNIC ACID—NO SALT.									
—	—	1·12	74	1·12	62	1·08	83	—	—
2·10	69	2·08	74	2·06	68	—	—	—	—
2·88	78	3·01	77	3·01	68	—	—	—	—
4·08	81	4·08	76	4·09	72	3·07	63	3·00	48
4·96	73	4·99	76	4·98	68	—	—	—	—
5·98	78	5·95	72	5·97	68	5·99	75	5·89	65
6·78	69	6·75	70	6·85	68	6·79	81	6·78	67
7·75	80	7·81	67	7·86	74	7·80	83	7·78	70
TANNIC ACID—0·5M SODIUM CHLORIDE.									
—	—	1·33	76	1·34	64	1·33	75	—	—
2·18	73	2·20	76	2·19	65	2·18	69	2·05	66
2·70	77	3·13	72	2·69	64	2·70	66	2·71	68
3·76	77	3·77	78	3·74	66	3·09	69	3·13	66
3·76	79	4·15	75	4·18	67	3·74	68	3·76	71
4·18	78	4·84	75	4·83	66	4·22	69	4·13	67
4·85	77	5·72	75	5·72	67	4·82	70	4·83	60
—	—	—	—	—	—	5·72	71	—	—
MIMOSA—NO SALT.									
1·53	74	1·51	81	—	—	—	—	—	—
2·20	78	2·16	84	—	—	—	—	—	—
2·77	83	2·78	84	—	—	—	—	—	—
4·16	86	4·19	83	—	—	—	—	—	—
5·01	87	5·06	82	—	—	—	—	—	—
6·03	84	5·87	81	—	—	—	—	—	—
6·92	84	6·95	82	—	—	—	—	—	—
7·85	85	7·74	81	—	—	—	—	—	—

(b) *Tannic Acid with the addition of* 0.5M *Sodium Chloride.*

The presence of 0.5M sodium chloride markedly affects the fixation of tannic acid by all the samples. Fixation by the untreated collagen is considerably decreased at low pH values, but is little affected above pH 4.0. The fixation of the hypochlorite-treated collagen and of the collagen methylated with methyl bromide is affected in a similar way but to a smaller extent. Fixation by the collagen methylated with methyl sulphate is only slightly reduced at low pH values, and is increased at pH values above 5.0, while fixation of the deaminated collagen is markedly decreased at all pH values.

There is even less variation in the shrinkage temperature than with the samples tanned without the addition of salt. Again, the shrinkage temperatures of the deaminated and hypochlorite-treated collagens are lower than that of the original collagen under corresponding conditions.

(c) *Mimosa without the addition of Sodium Chloride.*

The fixation of tannins from mimosa solutions by untreated, deaminated and hypochlorite-treated collagen is less than that from tannic acid solutions, but varies in a similar manner with pH except that there is only a slight indication of a maximum at pH 6. Similar curves for collagen and deaminated collagen were obtained by Thomas and Kelly[2], except that the maximum in the neighbourhood of pH 6 was more marked, and the decrease in fixation on deamination was much less.

Esterification of the carboxyl groups with methyl sulphate has a more marked effect on the fixation of mimosa than of tannic acid, the fixation

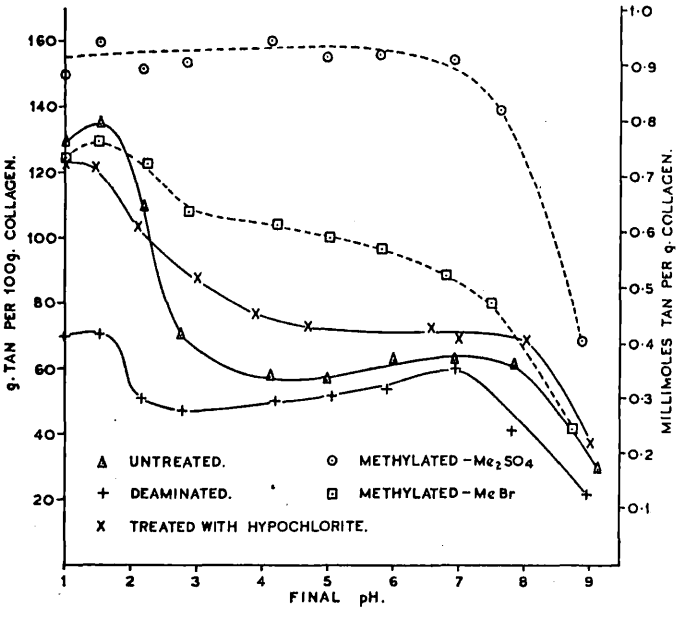

FIG. 3.

The fixation of mimosa tannin by collagen and modified collagen.

reaches its maximum at pH 7.0 and remains constant down to pH 1. The rather greater fixation of this modified collagen compared with the untreated collagen is probably due to the effect of the treatment on the fibre structure ; marked swelling of the pieces occurs during the methylating procedure and this will cause loosening of the structure.

Methylation with methyl bromide, which only esterified about half the carboxyl groups, had a similar but less marked effect.

The shrinkage temperatures of the samples tanned with mimosa are higher than those of the samples tanned with tannic acid under the same conditions. No shrinkage temperature determinations were carried out on the hypochlorite-treated and methylated collagens owing to lack of material.

(iii) Discussion.

It is difficult to draw definite conclusions from the results, and doubtless there are a number of ways of interpreting the experimental findings. The following observations, however, are worthy of note :—

1. Although tannic acid contains acidic groups[19, 31, 32] which can reasonably be expected to enter into salt formation with collagen, while mimosa does not, the mechanism involved in fixation appears to be the same with both tannins. The pH-fixation curves of collagen are similar for both materials except that mimosa does not show a second maximum in the region of pH 6, and modification of the amino, guanidino, and carboxyl groups has similar effects on the fixation of both materials.

2. The increase in fixation of tan as the pH falls from 4 to 2 appears to be related to the presence of amino groups, since removal of these groups almost completely eliminates this fixation. Treatment with hypochlorite, which modifies about 30% of the guanidino groups (see page 369), does not greatly affect fixation in this pH range. This might be expected since the guanidino groups are probably, in the main, converted to amino groups, and it is probable that guanidino and amino groups play a similar role in the fixation of tan. The treatment with hypochlorite is rather drastic, however, and other effects may also be operative.

3. Esterification of the carboxyl groups, leaving the basic groups charged, and thus giving the collagen a strong positive charge, causes the maximum tan fixation to be attained at higher pH values (pH 7.0 with mimosa) and to remain approximately constant over a wide pH range.

4. With the exception of methylation, modification of the reactive groups has less effect on fixation at pH values above 4 than at lower pH values. In the absence of added salt, there is little difference in the amount of tannic acid fixed by the different collagens at pH values above 6. With mimosa the differences are rather greater.

5. The addition of 0.5M salt decreases to varying extents the fixation of tannic acid by collagen and modified collagens at low pH values. This amount of salt almost eliminates acid swelling and it is possible that, in the absence of swelling, the effect of the various treatments on the structure of the collagen has an appreciable effect on the fixation of tan. The general decrease in the amount of tan fixed may be due to the effect of the salt on the charged groups

of the protein, or to competition between chloride and tannate ions for fixation by the collagen.

The results, as a whole, are consistent with the supposition that tan fixation is related to the positive charge carried by the collagen. With untreated collagen the fixation of tan is greatest between pH 1.5 and 2.0 when the carboxyl groups are all in the un-ionised form and the protein carries its maximum net positive charge. As the pH increases and the number of ionised carboxyl groups increases, thus reducing the net positive charge on the collagen, the fixation of tan decreases correspondingly, and at higher pH values, when the basic groups begin to lose their positive charge, the fixation of tan falls off rapidly.

Deamination, which decreases the number of positively charged groups on the collagen, decreases fixation, and treatment with hypochlorite, which also slightly reduces the number of positively charged centres, has the same effect. Methylation, by blocking the carboxyl groups and reducing the negatively charged groups to zero, causes the collagen to carry its maximum net positive charge over the whole stability range of the methyl groups, that is, from pH 2 to 7 or 8, and it is found that the fixation of mimosa by methylated collagen is constant and at a maximum over this pH range. The fixation of tannic acid by the methylated collagen shows a similar tendency but the effects are less marked.

TABLE IV.

ACID AND TAN FIXED BY COLLAGEN AND MODIFIED COLLAGEN.

(Millimoles per g.)

Sample	Acid-binding capacity	Tan fixed between pH 1·5 and 2 in the absence of added NaCl	
		Tannic acid	Mimosa
Untreated	0·88	1·0	0·80
Deaminated	0·44	0·50–0·55	0·41
Treated with hypochlorite ...	0·77	0·94	0·72

Assuming the molecular weight of tannic acid[33] and mimosa[34] to be 1700 (varying values have been obtained by different workers, but 1700 represents an average value), some interesting comparisons can be made between the amounts of tan fixed and the acid-binding capacities of the modified collagen[15] (see Table IV). The amounts of tan fixed by the different collagens between pH 1.5 and 2 are of the same order as their acid-binding capacities, suggesting that one molecule of tan is associated with each basic group. Especially striking is the relationship between the amounts fixed by untreated and deaminated collagen ; deamination halves the acid-binding capacity and also halves the fixation of both tannic acid and mimosa. Although the amounts of tan fixed are dependent on the concentrations of tan, time of tanning, and method of washing, it would seem reasonable to assume that there is some significance in these stoichiometric relationships.

The shrinkage temperature is dependent not only on the extent of tannage but also on the amount and acidity of the tan fixed[35], and in many cases these

factors may counteract one another. It may be concluded, however, that collagen tanned with mimosa has a higher shrinkage temperature than that tanned with tannic acid under the same conditions, presumably because of the lower acidity of the mimosa; and that deamination or treatment with hypochlorite tend to reduce the shrinkage temperature and to decrease the variations of shrinkage temperature with pH of tannage.

The Fixation of Chromium.

(i) Experimental Method.

The chrome tanning solution was prepared by the reduction of A.R. chromium trioxide (CrO_3) with sulphur dioxide : 34 g. chromium trioxide were dissolved in 500 ml. water and a current of sulphur dioxide passed through the cooled solution until excess was present. Free sulphur dioxide was then boiled off and the solution diluted to one litre. This solution was diluted with an equal volume of water to give the tanning solution, and was allowed to stand overnight before use. The final solution contained the equivalent of 13 g. Cr_2O_3 per litre in the form of chromium sulphate and was free from salts.

Collagen A, either untreated or modified, was used for all the experiments (for details see Table I).

Collagen in the form of pieces about 1 cm. square (2 g.) and a strip 1 cm. × 10 cm. (0.3 g.) for shrinkage temperature determinations, was soaked in water overnight, drained, and placed in 50 ml. of the chromium sulphate solution. After 24 hr. the pH was adjusted to the required value by the addition of sodium hydroxide, and maintained at this value by further additions of alkali at frequent intervals over a period of 48 hr. The pieces were left in the solu-

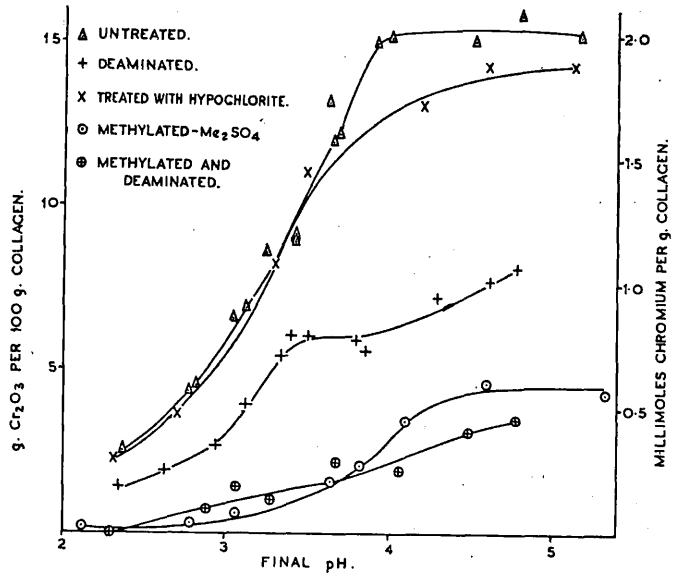

Fig. 4.

The fixation of chromium by collagen and modified collagen.

tion for a further 24 hr. (total time of tanning 4 days), drained, washed in a Wilson-Kern extractor with 8 litres distilled water over a period of 18 hr., and finally air-dried. The chromium content of the samples was determined by the method described by Davies and Innes[36] and the results expressed as g. Cr_2O_3 per 100 g. and as millimoles per g. of the original moisture and ash-free collagen (Fig. 4).

The shrinkage temperature determinations were carried out as before (see p. 371) except that all the samples were thoroughly wetted by immersion in water under reduced pressure before the determinations. When the shrinkage temperature exceeded 100°C. the determination was carried out in liquid paraffin, a stream of hot air being blown continuously round the side arm to aid in the circulation and heating of the paraffin. The determinations were carried out before the apparatus for determining the shrinkage temperature under pressure was completed[37], but it has since been found that the two methods give results which do not differ by more than a few degrees.

(ii) Experimental Results.

The amount of chromium fixed by collagen is not only dependent on the number of centres available for reaction, but on factors which affect the size of the chromium complex, that is, basicity and concentration of the liquor[38]. In the present experiment the concentration was kept constant and the effect of basicity was ruled out by covering a range of pH values.

With untreated collagen the chromium content increases sharply with rise in pH up to about 4.0 and then remains constant over the range covered (Fig. 4). The shape of the curve and the amounts of chromium fixed at any given pH value differ from those obtained recently by Bowes, Davies, Pressley, and Robinson[37] under similar conditions. This difference is probably due to the fact that in the earlier experiments the collagen was removed at the end of three days after the final adjustments of pH had been made, whereas in the present experiments the collagen was left in the solution for a further 24 hr., during which time the pH fell appreciably, thus causing the curve to be shifted to lower pH values.

Treatment with hypochlorite slightly decreases the maximum fixation and removal of the amino groups approximately halves it. Comparison with the titration curves of the modified collagens[15] shows that these decreases roughly correspond to two molecules of chromium for every basic group lost. Methylation with methyl sulphate and blocking of the carboxyl groups of the collagen decreases the fixation to an even greater extent than deamination. Chromium hydroxide was precipitated on the surface of these samples at high pH values and was difficult to remove, and it is probable that the recorded values are too high. It is also possible that some displacement of methyl groups by chromium takes place during tanning, and it would probably not be unreasonable to assume that complete inactivation of the carboxyl groups would reduce the fixation of chromium to negligible proportions. Deamination of the methylated collagen causes only a further small decrease in the chromium fixed.

The shrinkage temperature of the unmodified collagen increases progressively with the chromium content of the sample and reaches a maximum at 116°C. (Fig. 5). Although causing lower amounts of chromium to be fixed, treatment with nitrous acid or hypochlorite has no effect on the maximum shrinkage temperature attained. With the methylated collagen, however, the shrinkage temperature remained the same as that of the untanned collagen.

(iii) Discussion.

It is generally agreed that chrome tanning involves co-ordination of the complex chromium cation with suitable groups in the protein molecule, for example, carboxyl, amino, hydroxyl, or peptide groups. Consideration of the evidence at present available suggests that one of the most probable reactions occurring during chrome tanning is entry of the carboxyl groups of the collagen into the chromium complex in a manner analogous to that of the carboxyl groups of organic acids (see review by Bowes[39]). The results of this experiment strengthen this view and illustrate the essential part played by the carboxyl groups of the collagen, both with respect to the fixation of chromium and the attainment of a high thermal stability. Blocking of these groups by esterification with methyl sulphate reduces the fixation of chromium very considerably, and any chromium which is fixed has no effect on the shrinkage temperature.

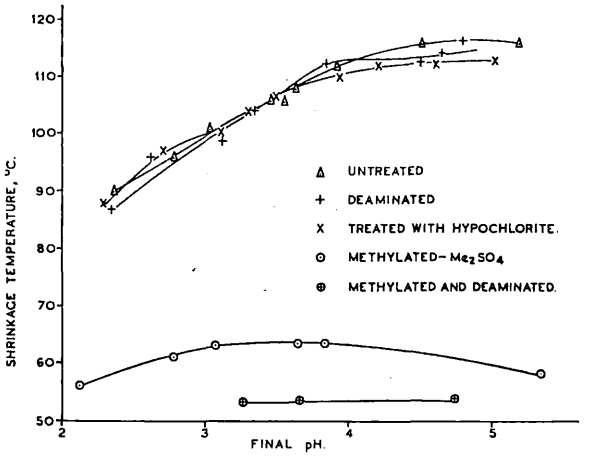

Fig. 5.

The shrinkage temperature of collagen and modified collagen after tannage with chromium sulphate.

The carboxyl groups will more readily enter into complex formation with chromium if present in the charged rather than the uncharged form, and it is suggested that the increased fixation of chromium with rise in pH, although partly the result of increase in the size of the chromium aggregates, is, in the main, due to the fact that as the pH increases the carboxyl groups progressively pass from the charged to the uncharged state. Gustavson[3] and Kuntzel and Riess[40] are also of the opinion that the increased fixation of chromium with rise in pH is mainly due to the effect of the pH on the collagen.

Treatment with nitrous acid also decreases the fixation of chromium to a marked extent, showing that amino groups must also play a part in chrome tanning. With methylated collagen, however, deamination causes only a very slight decrease in the fixation of chromium, the cause of which is uncertain. Thus, it may be concluded that chromium does not combine with amino groups independently of carboxyl groups, and the decrease found on the deamination of the original collagen must be due either to the effect of deamination on the reactivity towards chromium of other groups in the collagen, or to the fact that stable fixation of chromium requires co-ordination of the chrome complex with both carboxyl and amino groups (or other basic groups).

It is unlikely that the decrease in fixation is due to the first cause, for although deamination alters the iso-electric point of the collagen it does not affect the relative number of charged and uncharged carboxyl or other groups present at any given pH. Thus, the conclusion is reached that stable fixation of chromium requires co-ordination of both carboxyl and amino groups (or other basic groups) with the same chromium complex, that is, the fixation of the chromium complex at two points in the protein. Such a hypothesis has been put forward by various workers, for example, Kuntzel and Riess[41], to account for the high thermal stability of chrome-tanned leather, but these authors advanced no proof for this hypothesis other than the analogy with chromium-glycine complexes[41, 42]. (See also Gustavson[43].)

The fact that deamination does not affect the maximum shrinkage temperature attained may be attributed to the presence of sufficient guanidino groups to give the necessary number of cross-links to attain this stability.

The large decrease in fixation caused by blocking of the carboxyl groups, and the failure of any chromium that is fixed to influence the shrinkage temperature, preclude the possibility of the hydroxyl groups playing any significant part in chrome tanning as suggested by Elod and Schachowskoy[44, 45].

Although the validity of stoichiometric relationships between the amounts of chromium fixed and the reactive groups of the collagen may, in view of the complexity of the system, be open to question, it is of interest to note that the amount of chromium fixed between pH 4 and 5 approximately corresponds to the fixation of two molecules of chromium by each carboxyl group, and the decrease in fixation caused by treatment with nitrous acid corresponds approximately to two chromium molecules for each basic group (amino and guanidimo[15]) lost during the treatment. Evidence suggests that it is not improbable that the chromium aggregates in the tanning solutions used here contained, on the average, two chromium atoms[38].

Thus, the present work strengthens the evidence in favour of the hypothesis that chrome tanning involves co-ordination of the chromium complex with carboxyl and basic groups in the collagen molecules with the formation of cross-links between adjacent polypeptide chains.

The Fixation of Formaldehyde.

(i) Experimental Method.

Collagen in the form of pieces 1 cm. square (2 g.) and a strip 1 cm. × 10 cm.

(0.3 g.) was placed in 100 ml. of a 1% solution of 36% A.R. formaldehyde which had been previously adjusted to the required pH value by the addition of sodium hydroxide or hydrochloric acid. Tanning was allowed to proceed for three days, during which time the pH fell appreciably, the samples were then removed and washed in Wilson-Kern extractors with 8 litres of distilled water over a period of 24 hr. The last washing gave a very faint colour with Schiff's reagent. In a preliminary trial, a further washing with another 8 litres of water for 24 hr. was given, and the final wash water still gave a slight colour with Schiff's reagent. The decrease in the amount of formaldehyde fixed, however, was comparatively small and it was decided to standardise on a washing period of 24 hr. The samples were air-dried and left for a week before analysis.

The formaldehyde was determined by steam distillation with 40 ml. 2N sulphuric acid, about 450 ml. of distillate being collected[46]. The distillate was made up to 500 ml. and 200 ml. taken for precipitation with dimedone, following the method of Yoe and Reid[47] (see " Progress in Leather Science : 1920–1945 "[48]). This method of determination was compared with Romijn's method, which was employed in earlier investigations[8, 46], and was found to be more satisfactory for the present purpose where only small quantities of formaldehyde were involved.

The results are expressed as g. formaldehyde per 100 g. and as millimoles per g. of moisture- and ash-free collagen. The shrinkage temperature was determined in the same manner as that of the vegetable-tanned samples (p. 371).

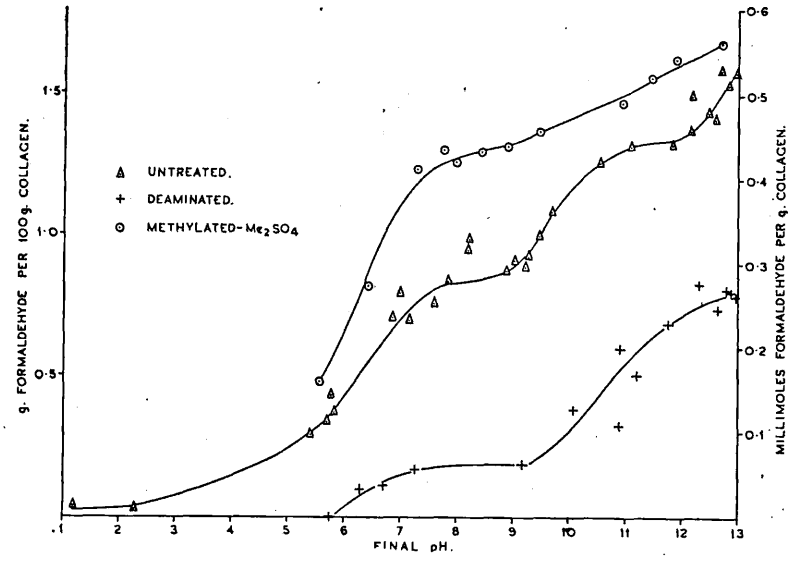

FIG. 6.

The fixation of formaldehyde by collagen and modified collagen.

(ii) Experimental Results.

Negligible amounts of formaldehyde are fixed by the original limed collagen at pH values below 2.0. As the pH rises, fixation increases, remains constant between pH 8 and 9, and then increases again as the pH is further increased (Fig. 6). There is an indication that the pH-fixation curve flattens slightly in the pH range 11 to 12. In all cases, the amounts fixed are lower than those found in a previous investigation[46] using the same method of tanning, probably because the washing procedure was more thorough. Even at the end of this more thorough washing procedure some formaldehyde was present in the last 20 ml. of wash water, and on washing for a further 24 hr., the formaldehyde was reduced by approximately 10%. Modification of the amino groups greatly reduced the fixation of formaldehyde between pH 2 and 9, but the increase in the amount fixed between pH 9 and 13 is unchanged.

TABLE V.

Shrinkage Temperature of Formaldehyde-Tanned Samples.

Collagen		Deaminated collagen		Methylated collagen	
Final pH of tanning solution	Shrinkage temperature, °C.	Final pH of tanning solution	Shrinkage temperature, °C.	Final pH of tanning solution	Shrinkage temperature, °C.
5·39	73	—	—	—	—
5·64	75	—	—	—	—
5·72	78	5·76	58	5·52	67
5·80	78	6·29	59	—	—
6·85	80	6·67	59	6·43	73
6·97	80	—	—	7·32	66
7·62	78	—	—	7·74	69
7·82	79	—	—	7·99	68
8·24	76	—	—	8·42	68
9·01	78	8·97	58	8·84	67
9·20	79	9·17	56	—	—
9·25	77	—	—	—	—
9·46	73	—	—	9·46	65
9·70	71	10·10	56	9·97	69
10·57	72	10·85	61	—	—
11·08	73	10·90	60	10·95	67
11·35	77	11·21	58	11·49	66
12·19	76	12·28	57	11·88	70
12·50	74	12·64	58	—	—
12·60	73	12·78	58	12·71	69
13·01	72	12·99	59	—	—

The fixation of formaldehyde by methylated collagen is greater than that of the original collagen at all pH values. It is unlikely that this increased fixation is caused by the blocking of the carboxyl groups, since at pH values greater than 9.0 the methyl groups are hydrolysed. A more likely cause is that for any given final pH the initial pH of the tanning solution was appreciably higher than that used for the original collagen, since allowance had to be made for acidity produced by the hydrolysis of the methyl groups.

The shrinkage temperature of the untreated collagen after treatment with formaldehyde varied from 73° to 80°C. (Table V). The fixation of quite small amounts of formaldehyde at pH values above 6.0 was sufficient to give the maximum shrinkage temperature (78°–80°C.); there was little change in shrinkage temperature as the pH rose from 6 to 9.2 and the formaldehyde fixed

increased from 0.35 to 0.90%, and at higher pH values the shrinkage temperature tended to decrease.

There was no obvious relationship between the variations in shrinkage temperature and the final pH value of the tanning solution. Certain differences were observed in the behaviour of the samples after the shrinkage temperature determination had been carried out. Samples tanned in solutions having a final pH below 5.7 did not pull out again after shrinkage ; as the pH increased the samples pulled out more readily, and between pH 7 and 12 pulled out almost to their original length ; but at higher pH values the samples became progressively harder and more difficult to pull out.

The shrinkage temperature of the deaminated collagen remained unchanged by the fixation of formaldehyde and, although more formaldehyde was fixed by the methylated collagen than by the original collagen at any given pH value, the shrinkage temperature was in all cases lower, varying from 65° to 73°C.

(iii) Discussion.

It is generally agreed that the main groups involved in the fixation of formaldehyde by proteins are the amino, guanidino and imidazole groups[49, 50] and it has recently been found that amide groups[50, 51, 52] and the indole groups[53] of tryptophane are also involved under some circumstances. It has been suggested that formaldehyde may also react with the peptide groups of the main polypeptide chain[51, 54, 55], but no direct evidence has been brought forward in support of this view, and experiments with polyglutamine[56] suggest that it cannot be a primary reaction though the possibility of secondary *condensation* of the peptide groups with formaldehyde molecules already fixed by other groups is not ruled out. Recent work by Fraenkel-Conrat and Olcott[52] suggests that reaction with amino groups and secondary condensation with amide groups plays an important part in the tanning and hardening action of formaldehyde.

The present work shows that, with collagen, the amino and guanidino groups are primarily responsible for the fixation of formaldehyde.

The flattening of the curve in the neighbourhood of pH 9.0 presumably marks the completion of the reaction of formaldehyde with the amino groups. Highberger and co-workers[9,13] found a similar flattening of the pH-formaldehyde fixation curve, and report that it is shifted to lower pH values as the concentration of formaldehyde is increased, a finding which is in accord with the evidence regarding the formol titration[56]. The further increase in the amounts of formaldehyde fixed as the pH rises from 9 to 13 is presumably due to reaction with the guanidino groups ; the curve shows little indication of flattening out, indicating that reaction with these groups is incomplete even at pH 13. (The guanidino groups in collagen do not titrate with alkali at pH values below 13.5[57], and it is possible that the pK of this group in collagen is considerably higher than has hitherto been realised.)

The amount of formaldehyde fixed by the original collagen up to pH 9.0, and the loss in fixation following deamination, correspond to considerably less

than the fixation of one formaldehyde molecule by each amino group. Similarly, the amount fixed from pH 9.0 to 13.0, the upper limit of pH covered, corresponds to the fixation of one molecule of formaldehyde by about half the guanidino groups.

The amounts of formaldehyde fixed at all pH values are appreciably less than those found by Highberger and co-workers[9-13], using a similar tanning technique and the same washing procedure. These workers determined the formaldehyde on the wet sample immediately after washing, whereas in the present investigations the samples were air-dried and left for a week before analysis, and it is possible that formaldehyde is lost during this time. Theis[58], using a pressing technique to remove excess formaldehyde, reports the fixation of considerably greater amounts of formaldehyde than other workers in this field. His procedure probably leaves a considerable amount of loosely bound formaldehyde (water removable), (see also Gustavson[59]); this would explain the high fixation he obtains and why he finds a much smaller decrease in fixation following deamination[12] than do other workers. These results emphasise the influence of the treatments succeeding tannage on the amount of formaldehyde held by the collagen, and suggest that more formaldehyde than has hitherto been realised can be reversibly held.

The maximum shrinkage temperature was attained by the fixation of comparatively small amounts of formaldehyde by collagen in the neighbourhood of pH 6. The importance of the amino groups with respect to the attainment of thermal stability is shown by the fact that the shrinkage temperature of deaminated collagen is unaffected by the fixation of formaldehyde. Similar results were obtained by Gustavson[60] with collagen, and Nitschmann and co-workers[55, 61] also found that the amino groups played an essential part in the hardening of casein with formaldehyde. (For a further discussion on the possible mechanisms involved in the attainment of thermal stability, see "Progress in Leather Science: 1920–1945"[48].) Although the guanidino groups fix an amount of formaldehyde comparable with that fixed by the amino groups, it has no effect on the thermal stability. It is possible that the formaldehyde reacts with two of the nitrogen atoms of the guanidino groups forming a cyclic compound, so that any possibility of a further reaction with another group in an adjacent chain, with the formation of a stabilising cross-link, is prevented.

Summary.

The fixation of tannic acid, mimosa tannins, chromium and formaldehyde by untreated, deaminated, hypochlorite-treated and methylated collagen has been studied.

The fixation of tannic acid and mimosa tannins is slightly reduced by treatment with hypochlorite and reduced to approximately one half by deamination. Esterification of the carboxyl groups with dimethyl sulphate or methyl bromide tends to increase fixation especially at pH values between 3 and 6; maximum fixation is attained at higher pH values and remains approximately constant at this value over a wide pH range. It is concluded that

fixation of vegetable tannins is related to the electrical charge carried by the collagen, being at a maximum when the net positive charge is greatest.

The fixation of chromium was slightly decreased by treatment with hypochlorite and reduced to about one half by deamination. These decreases correspond to approximately two atoms of chromium for each basic group lost during the treatments. Methylation almost entirely prevented fixation and the shrinkage temperature was not raised. Deamination and hypochlorite treatment did not affect the maximum shrinkage temperature attained by subsequent chrome tanning. It is concluded that the stable fixation of chromium involves co-ordination of both carboxyl and ammino (or other basic groups) of the collagen with the same chromium complex, thus forming a cross-link between adjacent polypeptide chains.

The fixation of formaldehyde by collagen is primarily due to reaction with the basic groups. The importance of the amino groups with respect to the attainment of hydrothermal stability was confirmed.

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Some Observations on the Amino-acid Distribution of Collagen, Elastin and Reticular Tissue from Different Sources

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By histological methods three types of connective tissue can be shown to be present in skins; collagen, elastic fibres and reticular tissue. Collagen, in the form of white bundles of interweaving fibres, makes up the greater part of the skin; the elastic fibres are pale yellow in colour and occur mainly in the grain layer; reticular tissue occurs around fat deposits and possibly as sheaths round the collagen fibre bundles (Dempsey, 1946). It is extremely difficult to isolate these three different tissues from skins. Tissues which stain similarly can be isolated fairly readily from other parts of the animal body: elastic fibres from the ligamentum nuchae (Vandegrift & Gies, 1901) and reticular tissue from lymph nodes (Bate-Smith, 1947) and from the large fat deposits (Maximow & Bloom, 1935; Dempsey, 1946), and some studies have been made on them. Before these tissues can be assumed to be identical with those occurring in skin, however, more conclusive evidence than staining reactions is required.

The aim of the present investigation was to determine what major differences in composition existed between the three main types of connective tissue, and to obtain information as to whether similar tissue preparations from different sources had the same composition.

EXPERIMENTAL

Analytical methods

Total N, amide N, amino N and titration curves were determined as described by Bowes & Kenten (1948*a*).

Preparation of samples

Ox-hide and alkali-treated sheepskin collagen were prepared as previously described (Bowes & Kenten, 1948*a*, *b*). Reticular tissue was prepared from lymph nodes and from the adipose tissue of ox. The wet lymph nodes were sliced, extracted with 5% (w/v) NaCl, washed and macerated with many changes of 30% ethanol in water. The fibrous mass was

dehydrated in 98% ethanol, extracted with light petroleum at 35° and air dried. Sheets of tissue from the fat deposit of an ox were mechanically freed from fat, extracted with light petroleum at 35°, washed with water and ethanol and dried in air.

The total N of the reticular tissues was rather lower than that of the collagen or elastin. The amide N was higher than that of elastin or of alkali-treated sheepskin collagen, and of the same order as ox-hide collagen (Table 1).

Table 1. *Analyses of connective tissues*

(Moisture and ash-free basis.)

Preparation	Total N (%)	Amide N (mmol./g.)	Amino N (mmol./g.)
Collagen (ox-hide)	18·6	0·66	0·46
Collagen (sheepskin, alkali-treated)	17·3	0·35	0·35
Elastin (ligamentum nuchae)	17·0	0·20	0·07
Reticular tissue (adipose tissue)	16·1	0·50	—
Reticular tissue (lymph nodes)	16·1	0·58	—

Elastin was prepared from the ligamentum nuchae of a freshly slaughtered ox. The tissue was freed from adhering matter, cut into approximately 0·5 cm. cubes, extracted with 10% (w/v) NaCl, washed free from salt, dehydrated with acetone and extracted with light petroleum (b.p. 40–60°) in a Soxhlet for 12 hr. The air-dried material (180 g.) was a pale yellow colour (N content 16·3%, moisture- and ash-free basis). To remove collagen, 168 g. of this material were autoclaved with 500 ml. water for 2 hr. at 120°. The supernatant liquor and the hot-water washings were combined and the total N extracted determined (see Table 2). (Increasing the time of autoclaving to 3 hr. caused no appreciable increase in the N extracted.) The autoclaved material was given eight further extractions of 1 hr. duration with 350 ml. portions of boiling water, and the total N removed in these extractions determined (Table 2). The elastin was dehydrated with acetone, again extracted with light petroleum and air dried (132 g.). Assuming that the residual material is elastin, the ligamentum nuchae after extraction with salt and removal of fat contained about 78% elastin and 22% collagen. This is in agreement with the value of 81% elastin found by Vandegrift & Gies (1901) and later by Lowry, Gilligan & Katersky (1941) for elephant ligamentum nuchae.

Table 2. *Soluble nitrogen extracted from ligamentum nuchae*

Extractions	Nitrogen extracted (% dry tissue)	(% total N)
During autoclaving	2·00	14·92
Subsequent extractions with water:		
First four	0·81	6·05
Fifth	0·066	0·49
Sixth	0·035	0·26
Seventh	0·008	0·06
Eighth	0·010	0·08
Total	4·921	21·86

The value for the total N of elastin (Table 1) is in agreement with previously determined values (Zoja (1897), 16·96; Richards & Gies (1902), 16·87; Stein & Miller (1938), 17·1%). The amide N, however, is appreciably lower than the two other values reported in the literature (Horbaczewski (1882), 0·41; Stein & Miller (1938), 0·38 mmol./g.); this is probably due to the fact that under the conditions used in earlier methods N, other than amide N, was returned as NH_3.

RESULTS

Titration curve of elastin

The titration curve of elastin with hydrochloric acid in the presence of 0·5 M-sodium chloride is given in Fig. 1, together with the corresponding curve for ox-hide collagen. The elastin shows an isoelectric point in the neighbourhood of pH 6. The curve shows a tendency to flatten between pH 2·0 and 1·5, but as the pH decreases further the amount of acid bound begins to increase again. This may be due to titration of fairly strong acid groups or of some weakly basic groups. The soluble nitrogen of the final solutions at pH values below 2·0 was only of the order of 0·5 mg./g. elastin, indicating that there was little solubilization of the protein. The curve indicates the presence of at least 0·2 mmol. basic groups per g. elastin.

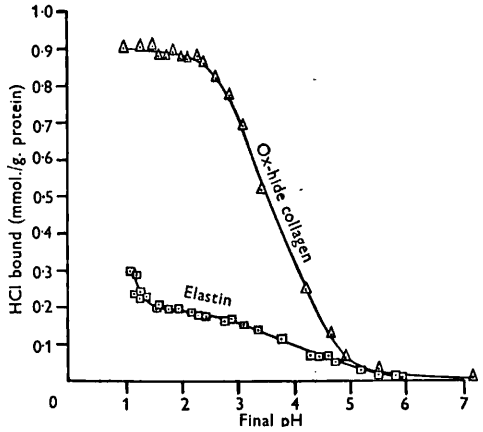

Fig. 1. Titration curves of elastin and ox-hide collagen in the presence of 0·5 M-NaCl.

Paper partition chromatography of samples

The technique described by Consden, Gordon & Martin (1944) was followed. The air-dry tissue (10±1 mg.) was hydrolysed with 0·5–1·0 ml. 6N-HCl at 100° for 24–30 hr. The hydrolysate was concentrated three times *in vacuo*, dissolved in 0·2 ml. water, and 10 μl. applied to a large sheet of Whatman no. 1 filter paper. The chromatogram was run in one direction with *s*-collidine in an atmosphere of diethylamine and HCN, and in the other direction with phenol in an atmosphere of NH_3 and coal gas. The chromatograms were examined immediately after spraying with ninhydrin, and

an estimation of the amino-acids present made from the size and colour intensity of the spots (Table 3). The strongest spots were arbitrarily assigned the value 9 and those just visible in a good light 1. Proline and hydroxyproline, which give yellow and orange colours respectively, were assessed as strong (S), medium (M) and weak (W).

Qualitative analyses by two-dimensional paper chromatographs of the reticular membrane from fat deposits and of elastin from ligamentum nuchae have been reported (Jordan Lloyd, 1946). In general the results agree, except that Jordan Lloyd reports the presence of hydroxyproline in elastin; this may be due to incomplete removal of the collagen. This author also reports that reticular tissue from fat deposits contains no tyrosine or hydroxylysine, whereas these were present in small amounts in our preparations. Consideration of the figures in Table 3 shows that elastin differs from collagen and both reticular tissue is converted to gelatin and a loose powdery substance which he terms 'reticulin' and which only slowly passes into solution. Both tissues lost approximately 50 % in weight, but while the chromatogram of the lymph-node tissue was not appreciably changed, that of the reticular tissue from the fat deposits showed a definite decrease in hydroxyproline. This suggests that this reticular tissue contained some collagen, and, in confirmation, the aqueous extract obtained on autoclaving formed a gel, while a similar extract from lymph nodes did not (see also Bate-Smith, 1947). On autoclaving for 18 hr. at 105°, 73 mg. of the air-dry reticular tissue from the fat deposits gave a residue of only 6 mg. This residue resistant to autoclaving might be expected to be elastin, but the paper chromatogram showed that this was unlikely since it contained appreciably more of the di-

Table 3. *The amino-acid distribution in some preparations of connective tissues as indicated by the relative intensities of spots on paper chromatograms*

Material ...	Collagen			Reticular tissues					Collagen residues	
				Lymph nodes		Fat deposits				
Source ...	Ox-hide	Alkali-treated sheep-skin	Elastin Ligamentum nuchae	Macerated	Auto-claved 4 hr.	Extracted with light petroleum	Auto-claved 3 hr.	Auto-claved 18 hr.	Ox-hide	Alkali-treated sheep-skin
Amino-acids:										
Alanine	7	7	9	8	8	9	8	8	6	9
Glycine	9	9	9	9	8	9	9	8	6	9
Leucine, etc.*	5	6	9	9	9	7	7	9	9	8
Phenylalanine	2	2	3	3	3	2	2	2	3	3
Valine	5	5	8	5	5	6	6	7	7	7
Cysteic acid	1?	1?	1	1	1	1	1	1	?	1
Serine	3	3	2	5	4	5	4	4	4	4
Threonine	2	2	2	4	4	3	3	4	3	3
Tyrosine	1	1	2	3	3	1	2	1	2	2
Arginine	5	5	1	6	6	4	6	3	4	3
Histidine	2	2	—	2	2	1	1?	—	2	1
Hydroxylysine	2	2	—	1	1	2	2	—	—	—
Lysine	5	5	1	6	7	4	5	5	5	3
Aspartic acid	5	6	3	7	7	5	6	5	7	4
Glutamic acid	7	7	4	8	8	7	9	6	8	6
Proline	S	S	S	M	M	M	M	M	M	M
Hydroxyproline	S	S	—	M	M	M	W	Trace	—	—

* Leucine, etc., refers to a composite spot which may contain leucine, isoleucine, and methionine.

types of reticular tissue in containing less arginine, lysine, hydroxyproline and glutamic acid, rather less aspartic acid, and rather more valine. In addition, it differs from the reticular tissues in containing rather less serine and rather more proline. Collagen differs from the reticular tissues in containing more proline and hydroxyproline. The reticular tissue from the fat deposits contains rather less of the dicarboxylic and basic amino-acids than the lymph node tissue.

It was considered possible that the reticular tissues were contaminated with collagen. The tissues were autoclaved for 3 hr. at 105°. This should remove collagen; but according to Siegfried (1902) carboxylic and basic amino-acids than elastin from ligamentum nuchae. It is possible that it is identical with Siegfried's 'reticulin'. Similarly, the residues (11 mg.) obtained on twice autoclaving ox-hide (2 g.) for 6 hr. at 105° followed by boiling for 1–2 hr., and the residue (30 mg.) obtained by boiling 4·76 g. limed sheepskin with 100 ml. 0·01 N-sodium hydroxide for 15 min., followed by repeated extractions with boiling water, gave paper chromatograms distinguishable from that of elastin by the presence of histidine and larger amounts of the dicarboxylic and basic amino-acids. The two residues differed slightly from one another, particularly in respect to the dicarboxylic acids, and from lymph-node reticular

tissue in containing no hydroxyproline. The absence of this amino-acid indicated the complete removal of collagen.

DISCUSSION

It is of interest to compare values given in the literature for the amino-acid composition of elastin with the results of paper partition chromatographic examination of the present preparation of elastin (Table 4). The chromatogram indicates that the values reported for aspartic acid and alanine are low, and that serine is present in approximately the same amount as threonine. The low figures for leucine and isoleucine obtained by Graham, Waitkoff & Hier (1949), using microbiological methods, suggest that the 'leucine fraction' of Table 4 may contain amino-acids other than leucine and isoleucine in comparatively large amounts. No hydroxyproline was detected in the chromatogram of the present preparation, and although it may not be entirely absent, the value of 2% given by Stein & Miller (1938) is possibly too high and may be due to incomplete removal of collagen. The low value for the amino nitrogen is in agreement with the small amount of lysine detected by analysis and by paper chromatography. The titration curve indicates that at least 0·2 mmol./g. of basic groups must be present in elastin; the amino nitrogen (0·07 mmol./g.), together with 0·06 mmol./g. of arginine (Stein & Miller, 1938), accounts for 0·13 mmol./g., leaving 0·07 mmol. of base to be accounted for. Histidine was not detected by paper chromatography, and consequently is present, if at all, in very small amount; it seems likely, therefore, that the discrepancy between the titration curve and analysis is due to a low arginine value.

Table 4. *Composition of elastin*

	Amino-acid (g./100 g.)	Amino-acid (N as % protein N)	Relative intensities of spots on paper chromatograms
Glycine	29·4 (*a*)	32·3	9
	25·75 (*b*)	28·3	
Alanine	6·85 (*b*)	6·3	9
Leucine fraction	30·0 (*a*)	18·8	9*
	21·38 (*b*)	13·4	
	10·7 (*h*)	6·7	
Valine	13·5 (*a*)	9·5	8
	13·8 (*h*)	9·7	
Phenylalanine	3·89 (*b*)	1·9	3
	3·34 (*c*)	1·7	
	4·8 (*h*)	2·4	
Tryptophan	0·0 (*a*)	—	—
Serine	—	—	2
Threonine	2·7 (*g*)	1·9	2
	1·1 (*h*)	0·8	
Cystine	0·23 (*a*)	0·2	(Cysteic acid = 1)
	0·6 (*h*)	0·4	
Methionine	0·38 (*a*)	0·2	—
	0·03 (*h*)	—	
Proline	15·2 (*a*)	10·9	5
	15·6 (*h*)	11·2	
Hydroxyproline	2·0 (*a*)	1·3	0
Arginine	1·0 (*a*)	1·9	1
	0·3 (*f*)	0·6	
	1·1 (*h*)	2·1	
Histidine	0·0 (*a*)	0·0	0
	0·04 (*h*)	0·1	
Lysine	0·0 (*a*)	0·0	1
	0·5 (*h*)	0·6	
Hydroxylysine	—	—	0
Aspartic acid	0·0 (*a*)	0·0	3
	0·6 (*b*)	0·4	
Glutamic acid	2·7 (*b*)	1·5	4
	3·3 (*h*)	1·8	
Tyrosine	1·6 (*b*)	0·7	2
	0·25 (*d*)	0·1	
	0·34 (*e*)	0·2	
	1·4 (*h*)	0·6	

* Isoleucine is present.

(*a*) Stein & Miller (1938). (*b*) Abderhalden & Schittenhelm (1904). (*c*) Kapeller-Adler (1932). (*d*) Horbaczewski (1882). (*e*) Schwarz (1893). (*f*) Kossel & Kutscher (1898). (*g*) Brand & Kassel (1942). (*h*) Graham *et al.* (1949).

Taking the highest figure for the 'leucine fraction' (30%) it is possible to account for approximately 90% of the total nitrogen of elastin. Judging from the chromatogram and the titration curve, about half this deficiency is due to the presence of more serine, arginine and aspartic acid than the present analytical figures suggest, leaving 5% still to be accounted for. It may be noted that Stein & Miller (1938) find that the sum of sulphur in methionine (0·08%), cystine (0·06%) and occurring as $SO_4^=$ (0·02%) agrees closely with the total sulphur of elastin (0·16%).

Comparison of the chromatograms suggests that elastin and reticular tissue from different sources may differ appreciably in composition. The residues from the exhaustive extraction of ox-hide, sheepskin and reticular tissue from fat deposits, which on the reported properties of collagen, elastin and reticular tissue, should consist mainly of elastin, do not give chromatograms similar to that of elastin from ligamentum nuchae, but, with the exception of the absence of hydroxyproline, give chromatograms more nearly resembling that of lymph-node reticular tissue. It appears, therefore, that the elastic fibres of skin are either appreciably different in composition from those of the ligamentum nuchae, or that they are less resistant to boiling water, and the residues obtained from skin consist of some other protein or proteins resembling reticular tissue in composition.

In view of the suggestions that have been made regarding the possible identity of reticulin and precollagen (Heringa & Weidinger, 1942) it is of interest

to note that with the exception of a lower proline and hydroxyproline content, the amino-acid distribution of the collagen and reticular tissue preparations are similar.

SUMMARY

1. Collagen has been prepared from ox-hide, elastin from ligamentum nuchae and reticular tissue from lymph nodes and fat deposits. Total nitrogen, and amide nitrogen have been determined and paper chromatograms of the hydrolysates examined.

2. Examination of the titration curve and chromatogram of elastin indicates that several amino-acids have still to be determined and that the reported values for arginine and alanine are low.

3. Tissues from different sources which stain similarly may vary appreciably in composition. With the exception of a lower proline and hydroxyproline content, the amino-acid distribution of the reticular tissues was similar to that of collagen.

4. Examination of chromatograms of the residues obtained by the exhaustive extraction of skin suggests that the elastic fibres of skin either differ from those of ligamentum nuchae with respect to amino-acid composition or are less resistant to hot water.

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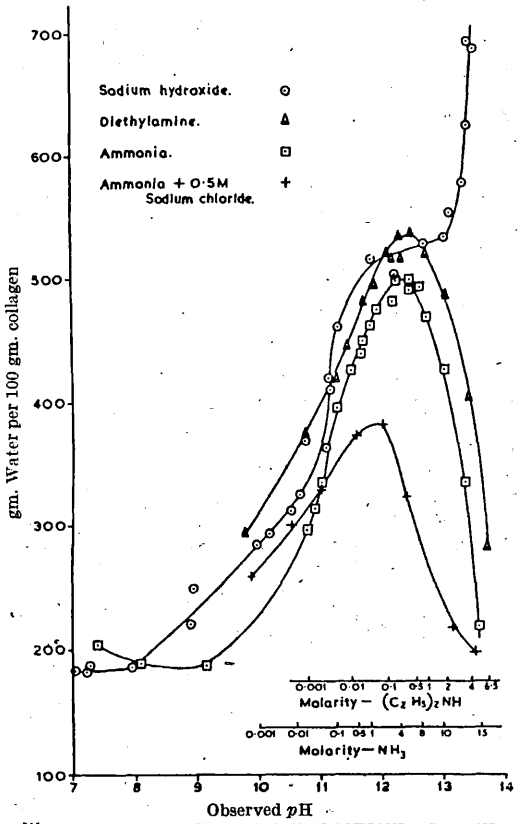
Uptake of Water by Collagen in Solutions of Alkalis and Strong and Weak Organic Bases

THE swelling of collagen and other structured proteins in solutions of strong acids takes place in accordance with the Donnan theory of membrane equilibrium[1,2,3]. Lyotropic effects of anions[4,5] and effects due to fixation of the anions by the protein[6,7,8] can also be observed.



WATER UPTAKE OF SKIN COLLAGEN IN AQUEOUS SOLUTIONS OF ALKALIS AT 20° C.

At pH values below about 3·0, the water uptake is greater with weak than with strong acids, and the decrease at lower pH values is no longer apparent[3,9]; this effect may be attributed to the action of the undissociated acid on the cohesion of the protein[9,10].

We find that swelling in alkaline solutions at 20° is more complicated. With the strong bases sodium hydroxide and tetramethyl ammonium hydroxide, the water uptake of skin collagen does not decrease at high pH values as predicted by the Donnan theory, but after increasing less rapidly with rise in pH in the range 11·5–13, it increases again sharply as the pH is further increased (see graph). (It has been established that the guanidine groups in collagen do not begin to titrate below pH 13·5 and hence cannot be responsible for this increase in water uptake.) With the weak bases ammonia and diethylamine, the water uptake changes over the whole pH range covered in accordance with the Donnan theory. (The observed pH values plotted in the graph were determined using the special Beckman glass electrode; when the concentration of base is high and the activity of water is appreciably less than 1, the observed pH values will begin to differ from the true values[11].)

These results cannot wholly be explained either on the basis of the Donnan theory, or of a specific cation effect. Although there is some indication that the sodium ion may have a specific effect in decreasing cohesion (see below), this effect is small compared with the effects now observed at high pH values; and the addition of sodium chloride does not alter the shape of the ammonia swelling-curve, but depresses the water uptake in accordance with the Donnan theory. It must be concluded, therefore, that the increased water uptake occurring at high values is associated with the presence of a high concentration of hydroxyl ions, which by some mechanism, probably breaking of hydrogen bonds, decreases the cohesion of the collagen and so allows it to take up more water. Since this effect is not operative with the weak bases, ammonia and diethylamine, it would appear possible that the presence of undissociated base in some way inhibits the action of the hydroxyl ions on cohesion.

We find that the addition of sodium chloride does not depress the water uptake in alkaline solutions to the same extent as in acid solutions, and conclude

that under these conditions the sodium ion or the salt itself decreases the cohesion of the collagen. On the addition of sodium chloride to calcium hydroxide solutions, we find that with concentrations up to 0·05 *M* the water uptake increases with increasing salt concentration, from 0·05 to 0·5 *M* it remains approximately constant, and at higher concentrations begins to decrease again. We conclude that at concentrations up to 0·05 *M* the water uptake increases owing to the effect of the monovalent sodium in increasing the osmotic swelling; at concentrations between 0·05 and 0·5 *M* this effect, and that of the salt on the cohesion of the collagen, is balanced by the effect of salt in depressing osmotic swelling; and at higher concentrations the water uptake is decreased by the dehydrating action of the salt.

We have also observed that under conditions which reduce the cohesion of collagen, for example, sodium hydroxide solutions above *p*H 13, the addition of organic solvents miscible with water, for example, acetone, *n*-propyl alcohol and dioxan, causes the collagen to disperse and to a large extent dissolve.

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The Swelling of Collagen in Alkaline Solutions

1. SWELLING IN SOLUTIONS OF SODIUM HYDROXIDE

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The swelling of collagen and other fibrous proteins in acid and alkaline solutions is governed by the osmotic pressure differences arising between the protein phase and the external solution as a result of the formation of protein salts—Donnan membrane effect—(Procter, 1914; Bolam, 1932; Donnan, 1933), and by the cohesion of the protein (Procter, 1914; Jordan Lloyd, 1920, 1938), i.e. the forces opposing swelling, such as interweaving of the fibres and intermolecular forces. In order to interpret the effect of pH changes on swelling it is, therefore, necessary to consider the influence of the acid or alkali both on the Donnan equilibria and on the cohesion of the protein.

The swelling of collagen and gelatin in alkaline solutions has received much less attention than that in acid solutions. A number of swelling curves of gelatin in alkaline solutions have been reported (Jordan Lloyd, 1920, 1930; Jordan Lloyd & Pleass, 1927, 1928; Loeb, 1921*a*, *b*, *c*, *d*; Kunitz, 1923–4; Pleass, 1930), but in general these do not extend above pH 12·0 and make no allowance for solution of the gelatin. A few alkaline swelling curves of collagen have also been reported (Kaye & Jordan Lloyd, 1924*a*, *b*; Jordan Lloyd, Marriott & Pleass, 1933; Highberger, 1936), but in all these there are insufficient points to determine the exact shape of the curves above pH 11·0. They show, however, no definite decrease in swelling at high pH values corresponding to that occurring in acid solutions below pH 2·0. With keratin (Jordan Lloyd & Marriott, 1934) and silk (Jordan Lloyd & Marriott, 1933; Coleman & Howitt, 1947) there is also an increase rather than a decrease in swelling at high pH values. With silk this increased swelling is accompanied by splitting up of the fibres, and is attributed to a weakening of the cohesive forces of the fibre.

The swelling of gelatin in alkaline solutions up to pH 10 is reduced by the addition of sodium chloride or other appropriate salts, in the same manner as swelling in acid solutions, but at higher pH values the curves have an upward trend, and the suppression of swelling is less marked (Jordan Lloyd & Pleass, 1927, 1928). There is also some evidence that the nature of the anion has some influence on swelling; e.g. sodium nitrate depresses swelling to a lesser extent than sodium chloride at the same molar concentration (Jordan Lloyd & Pleass, 1927, 1928). The few experiments which have been carried out indicate that salts do not suppress swelling of collagen in alkaline solutions to the same extent as swelling in acid solutions, nor to the same extent as swelling of gelatin in alkaline solutions (Kaye & Jordan Lloyd, 1924*b*).

Thus, it appears that the swelling of collagen in alkaline solutions is more complicated than in acid solutions, and that loss of cohesion of the collagen plays a more important part. The swelling of collagen in solutions of different bases, with and without the addition of salts, is, therefore, being investigated. The present paper deals with swelling in solutions of sodium hydroxide with and without the addition of sodium chloride.

EXPERIMENTAL

Raw material. Oxhide and alkali-treated sheepskin collagen D and E were prepared as described by Bowes & Kenten (1948*a*, *b*). The alkali-treated sheepskin was preferred for the present work, since, owing to its looser structure, it is able to take up more water and hence shows greater variations in swelling with changes in external conditions.

Swelling curves. Air-dry collagen (0·5 g.) in the form of pieces about 0·5 cm. square was placed in 100 ml. of the appropriate solution for 3 days at 20°. The pH of the solution was then determined and the pieces lightly blotted and weighed.

Preliminary experiments showed that, provided the pH was determined quickly, it was unnecessary to take any special precautions to avoid contamination with CO_2. The Cambridge glass electrode standardized with phthalate buffer (pH 4·0) and borate buffer (pH 9·22) was used for pH values up to 9·5 and the special Beckman electrode, standardized with Na_2CO_3-sodium borate buffer (pH 10·0) for pH values above 9·5. An electrode assembly mounted in a thermostat maintained at 20° (Coates, 1945) was used for the high pH values.

When the effect of time was being investigated, the pieces were removed, weighed, and returned to the solution again. During these experiments the pH fell slightly, probably owing to hydrolysis of the collagen and absorption of CO_2. This fall was small and insufficient to affect the conclusions drawn from the results.

For the swelling curve of gelatin, 0·05 g. of leaf gelatin, in pieces of 1 sq.cm. area were placed in 100 ml. of NaOH solutions of varying pH values. The solutions were previously cooled to 0° and kept at this temperature for 3 days. This temperature was chosen in preference to 20° in

order to minimize solution of the gelatin. The pieces of gelatin were removed and lightly wiped with blotting paper. The solutions were warmed to 20° and the pH values determined. The N contents of the majority of the solutions were determined by a micro-Kjeldahl method (Markham, 1942). Assuming that the N was all derived from solution of the gelatin (an assumption which will not be greatly in error except where low concentrations of N are involved), and taking the N content of the gelatin as 18%, the amount of gelatin which had dissolved was calculated. Allowance was made for dissolved gelatin in calculating the percentage water uptake of the gelatin.

RESULTS

The effect of time on swelling. The effect of time on the swelling of oxhide collagen in solutions of sodium and calcium hydroxides is shown in Fig. 1. The pH values of the solutions at the end of 3 and 18 days are recorded on the graph. The curves are similar in shape to those obtained by Jordan Lloyd (1920) with gelatin, and by Balfe, Beakbane & Wallis (1945) for collagen in acid solutions. There was a rapid uptake of water from all the solutions during

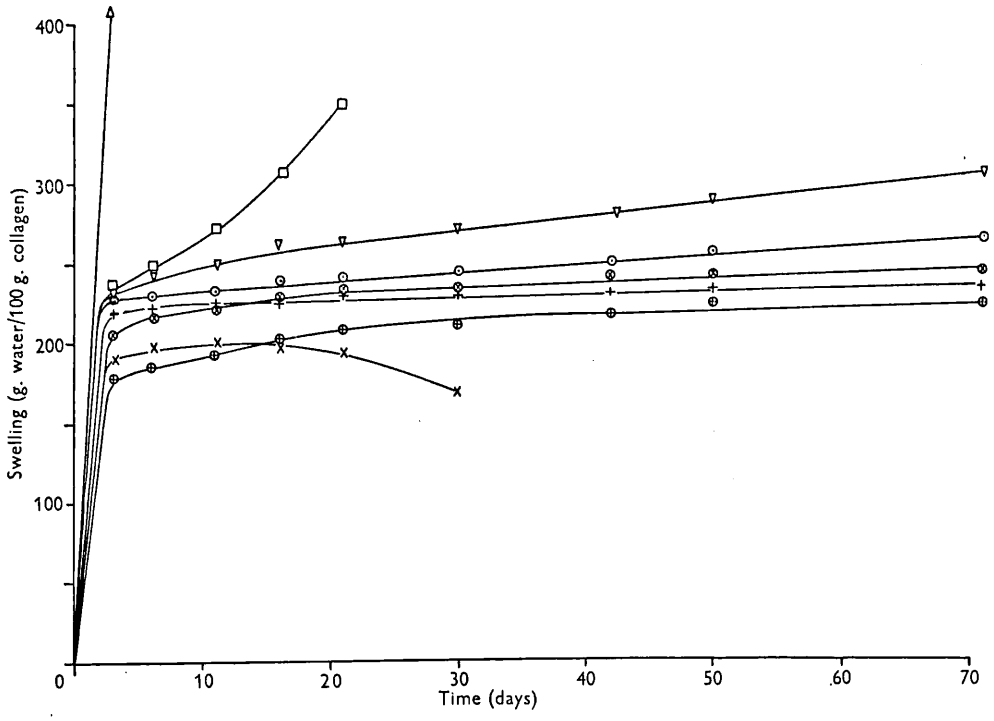

Fig. 1. The effect of time on the swelling of collagen in alkaline solutions.

	pH at 3 days	pH at 18 days		pH at 3 days	pH at 18 days
×, NaOH	11·34	10·80	△, NaOH	13·65	—
⊙, NaOH	12·84	12·83	+, NaOH + 0·1% Na_2S	12·72	12·67
▽, NaOH	13·05	13·02	⊕, Saturated $Ca(OH)_2$	12·66	12·66
□, NaOH	13·32	13·21	⊗, Saturated $Ca(OH)_2$ + 0·1% Na_2S	12·72	12·69

In all experiments swelling was taken as the amount of solution held expressed as a percentage of the weight of the moisture- and ash-free collagen or gelatin.

The pH values quoted refer to the pH at 20°. In alkaline solutions the pH decreases as the temperature increases, whereas the pOH, which is the important factor governing the swelling, remains unaltered. For this reason the solutions used in the determination of the swelling curve of gelatin were warmed to 20° before the pH was determined.

the first 2 days, followed, with one exception, by a further increase which was not complete at the end of 72 days. This further increase in water uptake became more pronounced as the pH increased. At pH 13·32 the curve rose quite sharply and after 21 days the collagen began to disintegrate. At 13·65, the highest pH value examined, the water uptake increased steadily and at the end of the fourth day the collagen was almost completely dissolved.

At the end of 3 days the swelling in excess of that occurring in the isoelectric range was half as great with calcium as with sodium hydroxide at the same pH, but as time elapsed swelling increased more rapidly in calcium than in sodium hydroxide solutions, so that at the end of 70 days there was little difference in the swelling of the two samples.

11·5 and 13·0 and then increased again very rapidly with further rise in pH. There is no evidence that the guanidino groups in collagen begin to titrate below pH 13·3 (Bowes & Kenten, 1948*a*), or that there is any large increase in the number of titratable groups during 3 days' exposure to pH values of this order (Bowes & Kenten, 1948*b*). Further, the amide N

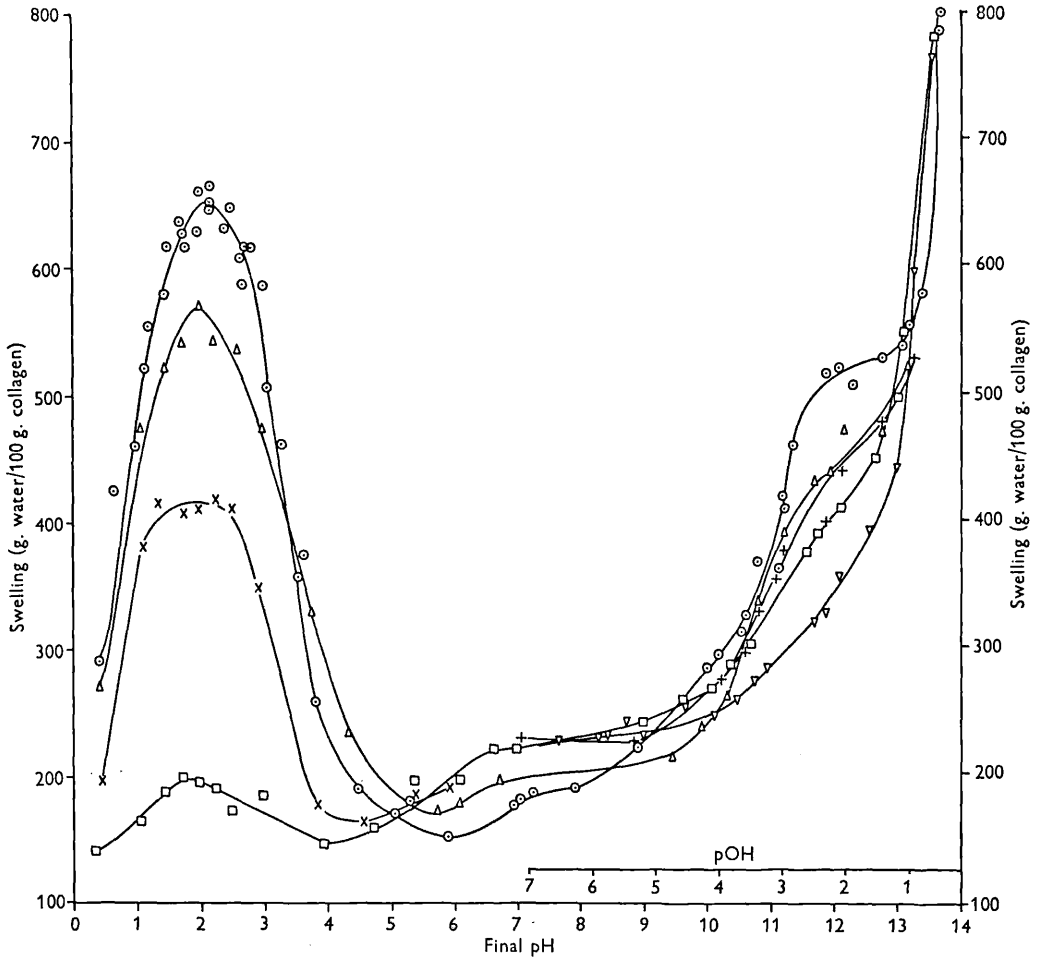

Fig. 2. Swelling of collagen D in hydrochloric acid/sodium chloride and sodium hydroxide/sodium chloride systems at 20°; ⊙, no salt; △, 0·05M-NaCl; +, 0·1M-NaCl; ×, 0·2M-NaCl; □, 0·5M-NaCl; ▽, 2M-NaCl.

The swelling of collagen in sodium hydroxide and sodium hydroxide/sodium chloride solutions. The swelling of alkali-treated sheepskin collagen in solutions of sodium hydroxide and hydrochloric acid over the pH range 0·5–13·5 is shown in Figs. 2 and 3. The curve for hydrochloric acid is included for purposes of comparison; it has the usual shape of such curves and calls for no special comment.

Swelling increased fairly sharply with rise in pH up to about 11·5, increased less rapidly between pH

of sheepskin E was only reduced from 0·39 to 0·22 mmol./g. on treatment in a sodium hydroxide solution at pH 13·4 for 3 days. It is unlikely, therefore, that this last rapid increase in swelling is due to increased formation of protein salt and hence to increase in the forces drawing the water into the collagen (swelling pressure). Thus, swelling in alkaline solutions differs markedly from that in acid solutions in showing no decrease at high concentrations as predicted by the Donnan theory.

In acid solutions swelling decreased progressively as the concentration of sodium chloride was increased, and at concentrations of 0·5M was almost completely suppressed (Fig. 2). In solutions of sodium hydroxide the effect of salt was much less marked; although there was an appreciable decrease in swelling in the presence of 0·05M-sodium chloride, further increase in the salt concentration had only a comparatively small effect and at pH values above 13·0 the effect was hardly perceptible (Figs. 2 and 3).

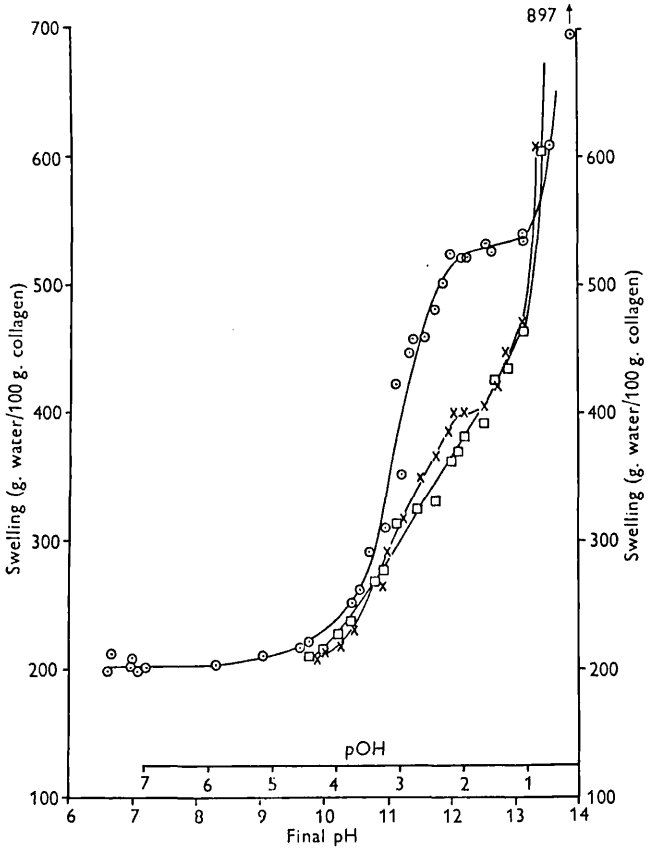

Fig. 3. Swelling of collagen E in sodium hydroxide/sodium chloride systems at 20°; ⊙, no salt; ×, 0·2M-NaCl; □, 0·4M-NaCl.

The swelling of gelatin in sodium hydroxide solutions at 0°. The increased swelling of collagen at high pH values may be due to breakdown of reticular tissue and elastin, to the removal of other structural factors restricting swelling, or to loosening of intermolecular forces such as salt links and hydrogen bonds. If the swelling curves of gelatin and collagen at high alkalinities are similar in shape, then it is probable that breakdown of intermolecular forces is the main cause of the decrease in cohesion, whereas if the curves differ in shape it suggests that, with collagen, breakdown of structural factors is also involved. The existing swelling curves of gelatin do not offer conclusive evidence on this point, and the swelling curve of gelatin was accordingly determined at 0°, this temperature being chosen in preference to 20° in order to minimize solution of the gelatin. Corrections were made for the gelatin dissolved (p. 2).

The swelling of gelatin (Fig. 4) increased with pH up to 12, decreased between pH 12·0 and 12·8, and then increased again rapidly with further rise in pH, until at pH values above 13·5 the gelatin disintegrated. There was a definite minimum at pH 12·7–12·8; hence it would appear that swelling takes place in accordance with the Donnan theory up to about pH 12·8 and only at higher pH values does some other factor come into play. This additional factor presumably involves the breakdown of intermolecular linkages, which leads to loss of cohesion and eventually to solution of the gelatin.

Consideration of the swelling curves in relation to the Donnan theory of membrane equilibria

From observation of the swelling curves alone it is difficult to estimate the extent to which water uptake diverges from that predicted by the Donnan theory, and hence to deduce the part played by decrease in cohesion in determining swelling. Swelling of fibrous proteins in acid and alkaline solutions is considered to be the result of the excess osmotic pressure (swelling pressure) in the protein phase arising from the difference in concentration of the diffusible ions in the protein and the external phase, equilibrium being reached when the osmotic pressure is balanced by the cohesive forces of the protein (Procter & Wilson, 1916). With gelatin, Proctor & Burton (1916) have shown that $e' = CV$, where e' is the difference in concentration of diffusible ions, V is the increase in volume of a given amount of gelatin, and C is a constant, which they term the 'bulk modulus' of the protein. For any given value of e' the increase in volume is dependent on C which is thus a measure of the forces restricting swelling, i.e. the cohesion of the protein. Cohesion here includes any force restricting swelling, such as interweaving of the fibres, network formation of reticular tissue or elastin and intermolecular forces such as salt links and hydrogen bonds. On the basis of the Donnan theory

$$x^2 = y(y+z),$$

and

$$e' = 2y + z - 2x,$$

where x and y are the molar concentrations of hydrogen or hydroxyl ions in the external and protein phases respectively, and z is the equivalent concentration of the protein anion or cation, whence

$$e' = -2x + \sqrt{(4x^2 + z^2)}.$$

Hence, e' and C may be calculated from a knowledge of the external pH, the acid or alkali bound by the protein and the increase in volume. With a fibrous protein, such as collagen, such calculations cannot be precise. Difficulty arises in allotting a value to z, since the volume available for solution in the internal phase is in doubt, and the increase in volume V is

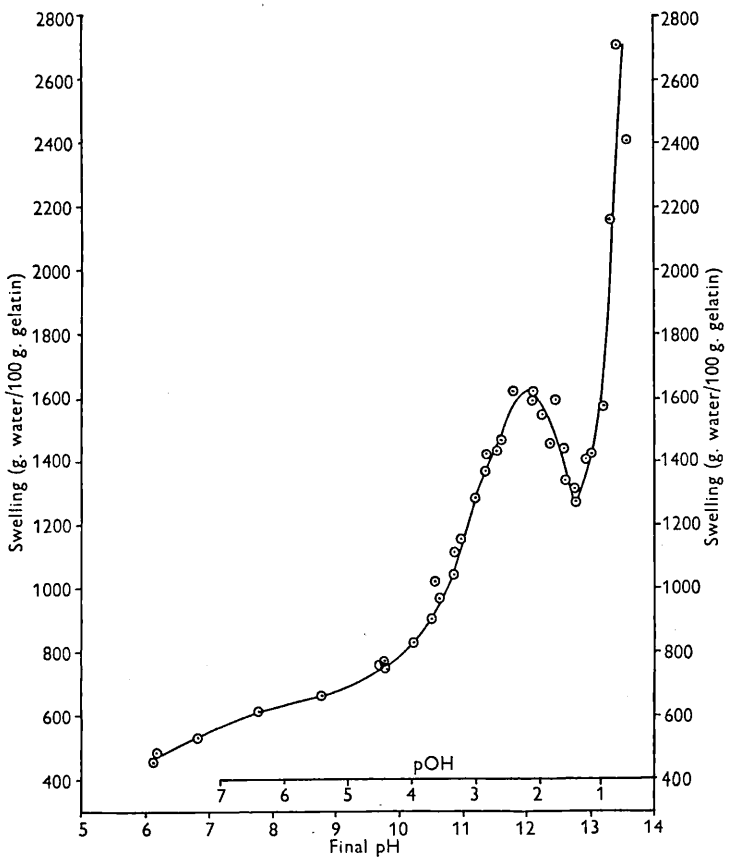

Fig. 4. Swelling of gelatin in solutions of sodium hydroxide at 0°.

difficult to determine accurately. In making the following calculations it has been assumed that all the water held by the collagen is available for solution and as an approximation the water uptake, in excess of that occurring in the isoelectric range, in ml./g., was taken as V.

On this basis e' and C were calculated for the following samples of collagen: (1) oxhide, using the swelling and titration curves obtained by Bowes & Kenten (1948*a*, *b*); (2) alkali-treated oxhide, using the swelling curves obtained by Balfe & Fowler (1948) and the titration curves of Retterova (1948); (3) alkali-treated sheepskin, using the present curves and the titration curves of Bowes & Kenten (1948*b*).

These calculations show that e' decreases as the pH increases above 12·0, e.g. with sheepskin collagen the value of e' decreases from 0·056 at pH 12·0 to 0·005 at pH 13·5. It follows, therefore, that the increased water uptake at high pH values cannot be due to an increase in swelling pressure, but must be attributed to a decrease in cohesion.

Typical values of C under various conditions are given in Table 1. For any one sample the values of C are approximately constant between pH 1 and 3 and between 10·5 and 11·0, the values being slightly lower in alkaline solutions than in the corresponding acid solutions. Above pH 12·5 the values of C decrease sharply. C is not affected by additions of sodium chloride up to 0·1 M, but at higher concentrations it begins to decrease and with 0·5 M-sodium chloride it is reduced to about one-quarter of the value found in the absence of salt. The value of C for alkali-treated collagen is slightly less than that of untreated collagen, showing that such treatment reduces cohesion. The values for the alkali-treated sheepskin collagens are only about one-fifth of those of the alkali-treated oxhide collagen under corresponding conditions. The fibres of sheepskin are much less closely woven than those of oxhide, thus offering less resistance to swelling, and the large difference in the value of C for the two types of skin indicates that the interweaving of the fibres must play a large part in determining cohesion. The corresponding value of C for gelatin in hydrochloric acid solutions is 0·00021 (Procter & Burton, 1916), i.e. 100 times smaller than the value for alkali-treated sheepskin.

Table 1. *Typical values of C, Procter's 'bulk modulus'*

($C = e'/V$, where V is the increase in volume of the protein in ml./g. and e' is the difference in concentration between the protein and the external phase in mol./l.)

	Sodium chloride concentration (M)					
Oxhide:	0·0	0·05	0·10	0·2	0·4	0·5
Acid solutions	0·127	—	—	—	—	—
Alkaline solutions, pH 10–12·5	0·075	—	—	—	—	—
Alkaline solutions, pH 13·5	0·008	—	—	—	—	—
Alkali-treated oxhide:						
Acid solutions	0·111	—	—	0·122	—	Large
Alkali-treated sheepskin D:						
Acid solutions	0·019	0·024	—	0·026	—	Large
Alkaline solutions, pH 10–12·5	0·013	0·016	0·013	—	—	0·003
Alkaline solutions, pH 13·5	0·001	0·005	0·002	—	—	<0·001
Alkali-treated sheepskin E:						
Acid solutions	0·021	—	—	—	—	—
Alkaline solutions, pH 10–12·5	0·020	—	—	0·016	0·010	—
Alkaline solutions, pH 13·5	0·002	—	—	<0·001	<0·001	—

DISCUSSION

This investigation shows that decreases in cohesion must play a much more important part in determining the swelling of fibrous proteins in alkaline solutions than in acid solutions. This can be qualitatively deduced from the shape of the curves and is borne out by mathematical analysis of the results on the basis of Procter's theory.

On the assumption that the value of C is an approximate measure of cohesion, it may be concluded that the cohesion of collagen in alkaline solutions is less than in acid solutions at corresponding pH values. The effect is small at pH values up to 12·5, but becomes increasingly marked at higher pH values, the value of C decreasing nearly tenfold between pH 12·5 and 13·5. This decrease in cohesion should eventually lead to solution of collagen and this in practice does occur, if the pH and the time of treatment is sufficiently long (p. 2). In this connexion it may be noted that collagen may be left in a suspension of calcium hydroxide (pH 12·6 at 20°) for long periods without obvious damage, but on subsequent warming the collagen is much more readily converted to gelatin.

The presence of sodium chloride at concentrations of 0·2 M or greater also appears to decrease cohesion, with 0·5 M-sodium chloride the effect being equivalent to a 25% decrease in C. Salts are known to affect the swelling of proteins in the isoelectric

range, but in general the effect appears to be smaller than at higher pH values, and takes place at higher concentrations of salt (cf. Page & Page, 1927). Kunitz (1929–30) finds that the elasticity of gelatin in the isoelectric range is only appreciably affected when the salt concentration exceeds 0·8 M.

The reason why salts almost completely suppress the swelling of gelatin in alkaline solutions, while only having a comparatively small effect on the swelling of collagen, is related to the much larger uptake of water by gelatin. Because of this the concentration of diffusible ions in the gelatin phase is comparatively low, and hence the difference in concentration between the internal and external phases is reduced to negligible proportions by quite low concentrations of salt which have no effect on cohesion.

The first rapid swelling observed when collagen is placed in alkaline solutions is probably due to osmotic effects. The further slow increase taking place over long periods of time may be due to combination of base with additional groups liberated as a result of hydrolysis of peptide and amide bonds and the consequent formation of an increased amount of protein salt; or more probably, in view of the limited chemical changes of this kind which take place (Bowes & Kenten, 1948*b*), to a decrease in the cohesion of the collagen. Since the further increase in swelling is more marked with calcium than with sodium hydroxide at the same pH value, the calcium ion appears to be more effective in decreasing cohesion than the sodium ion.

The decrease in cohesion observed in alkaline solutions is apparently not primarily associated with breakdown of the main polypeptide chains, since apart from some loss of amide nitrogen and hydrolysis of a very small number of peptide links there is little chemical change in the collagen (Bowes & Kenten, 1948*b*). Further, since a prolonged alkaline soak is an important pretreatment in one method of preparation of gelatin, the collagen presumably eventually goes into solution in the form of large polypeptides and not as peptides. Comparison of the swelling curves of collagen and gelatin suggests that the increased swelling of both proteins at high pH values is primarily due to the same cause, probably breaking of intermolecular links such as hydrogen bonds. However, the existence of a minimum in the pH range 12·0–12·8 with gelatin, but not with collagen, suggests that with collagen breakdown of structural features restricting swelling, such as reticular tissue, elastin, etc., may also be involved.

It is uncertain how far the effects on cohesion observed in alkaline solutions are permanent and influence the subsequent behaviour of the collagen. Some effect certainly remains, since the increase in swelling in the acid or isoelectric range following alkaline treatment is greater than can be accounted for, on the basis of an increase in the number of reactive groups (Bowes & Kenten, 1948*b*). Provided the decrease in cohesion is not too great and the polypeptide chains are not forced too far apart, it would appear reasonable to assume that the process can largely be reversed, the intermolecular links between the chains reforming as water is withdrawn.

SUMMARY

1. The swelling of collagen in sodium hydroxide solution increases progressively with increase in pH, and shows no decrease at high pH values corresponding to that occurring in acid solutions below pH 2·0.

2. The addition of small amounts of sodium chloride (0·05 M) decreases swelling at pH values up to 12·5, but at higher pH values has little effect. Higher concentrations of salt cause little further decrease in swelling.

3. Consideration of the curves in relation to the Donnan theory indicates that decrease in cohesion plays an important part in determining swelling in alkaline solutions. There is some decrease in cohesion at all pH values above 11·0, but more especially above 13.

4. Sodium chloride tends to decrease cohesion.

5. Decrease in cohesion is in part due to breakdown of structural features, but in the main to breaking of intermolecular links.

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The Swelling of Collagen in Alkaline Solutions

2. SWELLING IN SOLUTIONS OF UNIVALENT BASES

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In Part 1 (Bowes & Kenten, 1950) it was shown that changes in cohesion as well as osmotic effects must play an important part in determining the swelling of collagen in solutions of sodium hydroxide. It seemed probable from these results that the increased swelling at high pH values was due to a decrease in the cohesion of the collagen, and that this decrease in cohesion was associated with the presence of a high concentration of hydroxyl ions rather than with any specific effect of the sodium ion. It has been suggested, however, that bases differ in their effect on collagen; e.g., ammonia is reported to cause more splitting up of the fibres (an indication of decreased cohesion) than sodium hydroxide (Kaye & Jordan Lloyd, 1924).

The swelling of collagen in solutions of a number of strong and weak bases has, therefore, been studied to gain information regarding these effects, and, in particular, with respect to the nature of the decrease in cohesion occurring at high pH values in solutions of sodium hydroxide. In a brief account of the preliminary work (Bowes & Kenten, 1947) it was shown that, in contrast to strong bases, the swelling of collagen in solutions of weak bases decreased at high pH values in apparent accordance with the Donnan theory. It was suggested that the undissociated base in some way inhibited the action of the hydroxyl ions on cohesion. By comparison with swelling curves obtained in the presence of organic solvents it has now been shown that the decrease is related to the progressive departure from an aqueous medium as the concentration of base increases.

EXPERIMENTAL

Materials

Collagen. The alkali-treated sheepskin collagen D and E described in Part 1 (Bowes & Kenten, 1950) was used.

Guanidine. The free base was prepared from the sulphate by the addition of a slight excess of $Ba(OH)_2$ followed by removal of excess Ba^{++} by the addition of H_2SO_4. The solution so prepared gave no test for SO_4^{--}, and the ash content was 0·0012 g./100 ml.

Tetramethylammonium hydroxide. An aqueous solution of tetramethylammonium bromide prepared from trimethylamine and methyl bromide (Hickinbottom, 1948) was treated with Ag_2O, and the solution filtered. The resulting solution was 0·66N.

Diethylamine. Technical diethylamine was shaken with charcoal, dried over solid NaOH and the free base distilled off.

The other bases and organic solvents were either of A.R. or technical grade as supplied by British Drug Houses Ltd.

Determination of swelling curves

The technique adopted was the same as that used in Part 1 (Bowes & Kenten, 1950). The weight of water or solution held by the collagen, expressed in terms of percentage of the moisture- and ash-free weight, was again taken as a measure of swelling. No special precautions were taken to avoid contamination with CO_2 or loss of base by volatilization, but the time during which the solutions were exposed to the atmosphere was kept to a minimum.

With the weak bases, changes in pH with concentration were small, and, in addition to pH measurements, the final concentration of base was determined by titration. In some cases it was obvious that appreciable amounts of collagen were dissolved, but except when using acetone and dioxan, it was not possible to check this by N determinations owing to the high N content of the bases. Where appreciable solution of the collagen was suspected, the samples were air-dried, heated in an air oven at 106° for 6 hr. and the percentage loss in weight, based on final weight, taken as a measure of the swelling. This method of calculating the results is not ideal, since appreciable amounts of base may be retained by the collagen after drying and heating under alkaline conditions may lead to some decomposition of the collagen.

All pH values were determined at 20° and pH values quoted refer to the values at 20°. The Cambridge glass electrode standardized with phthalate buffer (pH 4·00) and borate buffer (pH 9·22) was used for pH values below 9·5, and the special Beckman electrode standardized with Na_2CO_3-$Na_2B_4O_7$ buffer (pH 10·00) for the pH values above 9·5. There is some doubt regarding the pH values of the solutions containing high concentrations of weak bases or organic solvents. The pH values may be in error owing to the limitations of the glass electrode in alkaline solutions (Dole, 1941*a*). This error is probably small in solutions of organic bases (Dole, 1941*a*; Hill, 1929; Hubbard, Hamilton & Finn, 1939) and will cause the observed pH to be lower than the true value. McInnes & Dole (1930) found little error with 0·1N solutions of tetramethylammonium hydroxide. Deviation of the activity of water from unity may also introduce errors, but by analogy with similar effects in acid solutions and solvent water mixtures these are also likely to be small (Dole, 1941*b*).

It is the hydroxyl-ion activity which governs swelling, however, and although the observed pH values may not be greatly in error, alterations in K_w and in the activity of water

will affect the pH-pOH relationship, and hence the position of the swelling curves relative to one another. For true comparison with the NaOH curves, those of the weak bases should be progressively shifted towards the left as the concentration of base increases. Judging from the values of K_w in dioxan and water mixtures (K_w decreases from $0{\cdot}6809 \times 10^{-14}$ to $1{\cdot}234 \times 10^{-16}$ as the concentration of dioxan increases from 0 to 45%; Harned & Fallon, 1939), and from the change in pH of 0·01 N-NaOH solutions on the addition of acetone (Table 1), this shift should be of the order of one pH unit when the concentration of base is increased from zero to between 30 and 40%.

RESULTS

Swelling curves in solutions of strong and weak univalent bases

The swelling curve of collagen in tetramethylammonium hydroxide solutions was almost identical with that in sodium hydroxide solutions (Fig. 1);

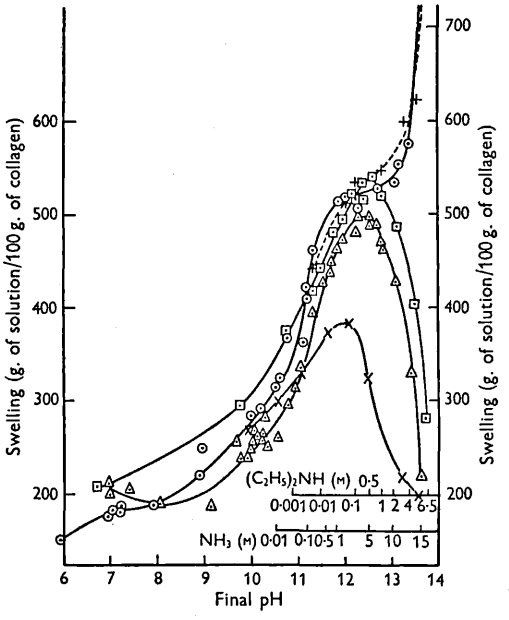

Fig. 1. Swelling curves of collagen in solutions of NaOH, ⊙—⊙; tetramethylammonium hydroxide, + --- +; diethylamine, ⊡—⊡; NH_3, △—△; NH_3 with the addition of 0·5 M-NaCl, × — ×.

swelling increased rapidly with pH up to 11·5, increased more slowly in the range 11·5–13·0, and then increased rapidly with further increases in pH. The curve for guanidine (Fig. 2) was of the same shape, but swelling was less than in sodium hydroxide solutions at pH values up to 13·5. The smaller amount of swelling at pH values below 13·5 is probably due to combination of guanidinium ions with the collagen so that they make no contribution to the swelling pressure.

The swelling curves of collagen in solutions of the weak bases (Figs. 1 and 2) varied only slightly from that in sodium hydroxide solutions up to about pH 12·5, but at higher pH values they began to show considerable divergencies, the swelling decreasing with pH in apparent agreement with the Donnan Theory, until at about pH 13·5 it approached that found in the isoelectric range. The decreased swelling is not due to disintegration of the collagen, since the pieces in the most concentrated solutions were obviously unswollen. Also on drying there was no appreciable loss in weight of the collagen. Although variations in the pH-pOH relationship with increasing concentration of base give rise to some doubt regarding the exact position of the curves for the weak bases in relation to the curve for sodium hydroxide (p. 524), such variations do not alter the fact that the swelling decreased with increasing concentration of base. The addition of 0·5 M-sodium chloride decreased swelling in solutions of ammonia at all pH values, but the shape of the curve was

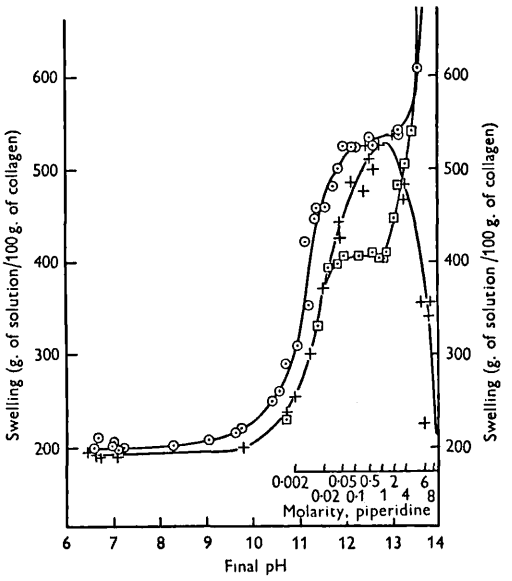

Fig. 2. Swelling curves of collagen in solutions of NaOH, ⊙—⊙; guanidine, ⊡—⊡; piperidine, + — +.

unchanged. At pH 12·0 the decrease in swelling was of the same order as that in sodium hydroxide solutions at equivalent sodium chloride concentrations.

These findings rule out the possibility that the decrease in cohesion observed at high pH values in sodium hydroxide solutions is due to a specific effect of the sodium ion and tend to confirm the view that it is associated with high concentrations of hydroxyl ions which by some mechanism, probably breaking of hydrogen bonds, reduce the cohesion of the collagen. The reason why this effect is apparently

not operative in solutions of weak bases was not at first clear. The most likely explanation appeared to be that the undissociated base inhibited the effect of the hydroxyl ions on cohesion. If this were so, then it might be expected that the molar concentration of the base would be a major factor in determining the effect.

The effect of the concentration of organic base at high pH *values*

To test the suggestions made above, the swelling of collagen in sodium hydroxide solutions containing varying amounts of ammonia was determined. The ammonia concentration was kept constant at 3·8, 7·6 and 10·6 N and the pH was varied by the addition of sodium hydroxide (Fig. 3). There was a considerable scatter of points, but the results indicated that at any given ammonia concentration, the amount of swelling was at first unaffected by rise of pH, but

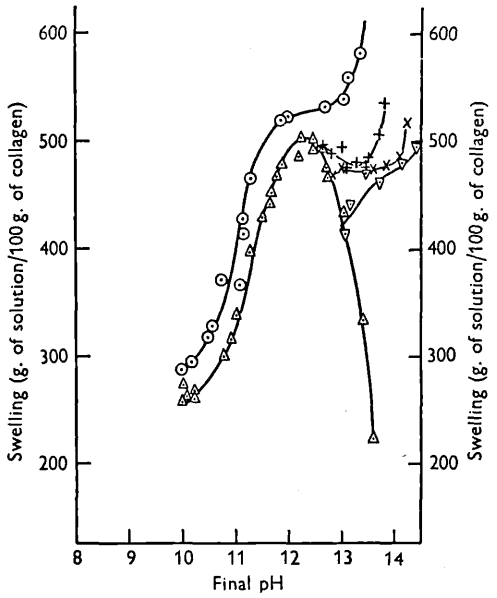

Fig. 3. The effect of additions of NH_3 on the swelling curve of collagen in NaOH solutions. NaOH, ⊙—⊙; NH_3, △—△; NaOH + 3·8 M-NH_3, +—+; NaOH + 7·6 M-NH_3, ×—×; NaOH + 10·6 M-NH_3, ▽—▽.

finally increased in the same way as with sodium hydroxide alone. Apparently, the higher the pH, the greater the concentration of weak base necessary to suppress swelling. Consideration of the curves in Figs. 1–3 indicates that at equivalent molar concentrations diethylamine was much more effective than ammonia, and slightly more effective than piperidine in suppressing swelling. The effect of a number of different bases at the same molar concentration over a range of pH values was, therefore, determined, using 5 M-solutions of the bases, adjusted to the required pH's with sodium hydroxide or hydrochloric acid. With all the bases swelling increased with pH, but the relative effects varied greatly (Fig. 4). There was an indication that the effectiveness of the bases in suppressing swelling was related to their concentration in g., but some specific effect was also apparent; e.g. diethylamine had a much greater effect than other bases at the same or greater concentrations. It was also found that the swelling curve in 36 % (v/v) acetone solutions was not very different from that found with pyridine and some of the other weak bases (Fig. 4).

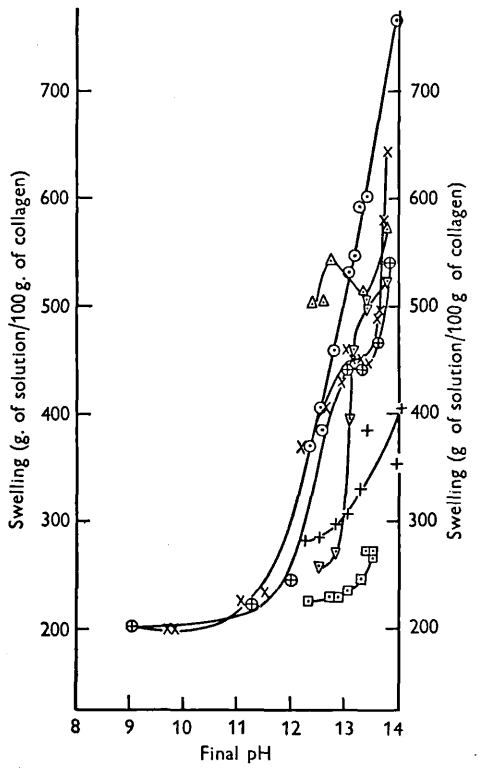

Fig. 4. The swelling of collagen in 5 M-solutions of NH_3, △—△; monomethylamine, ⊙—⊙; dimethylamine, ▽—▽; diethylamine, ⊡—⊡; pyridine, ×—×; piperidine, +—+; acetone, ⊕—⊕.

The effect of acetone and dioxan on swelling

To obtain further information on the influence of the medium on swelling, the effect of increasing concentrations of acetone and dioxan on the pH and the swelling properties of 0·01 and 0·1 N-sodium hydroxide solutions was determined. The addition of acetone progressively increased the pH (Table 1) due to alterations in K_w (p. 525), the shift in pH being of the order of 1·1–1·5 units in 50 % (v/v) acetone. The shift in pH with increasing dioxan concentration was much less, about 0·7 pH unit; this may have

Table 1. *The effect of acetone and dioxan on the* pH *values of sodium hydroxide solutions and the effect of acetone on the soluble nitrogen derived from collagen after soaking for* 3 *days in sodium hydroxide solutions at* 20°

(The time elapsing between the addition of the dioxan and the determination of pH varied from 0·5 to 1 hr.)

	Acetone in 0·01 N-NaOH			Acetone in 0·1 N-NaOH			Dioxan in 0·01 N-NaOH	
	pH			pH			pH	
Concentration of acetone or dioxan (ml./100 ml.)	Initial	After 3 days' contact with collagen	Soluble N (mg./g. collagen)	Initial	After 3 days' contact with collagen	Soluble N (mg./g. collagen)	Initial	After 3 days' contact with collagen
0	12·05	11·98	3·8	12·97	12·99	9·2	12·06	12·02
5	—	12·10	2·3	13·09	13·09	9·4	12·08	12·05
10	12·28	12·21	6·1	13·18	13·19	10·5	12·22	12·08
15	12·38	12·34	4·2	13·36	13·33	7·8	12·12	12·10
20	—	12·45	3·8	13·44	13·47	9·1	12·28	12·12
25	12·68	12·58	5·1	13·56	13·59	9·6	12·38	12·13
30	12·75	12·72	12·2	13·67	13·67	13·0	12·46	12·10
35	12·99	12·86	9·0	13·78	13·81	14·3	12·50	12·06
40	13·12	13·02	9·3	13·97	13·92	16·5	12·60	11·93
45	13·31	13·20	5·4	14·06	14·01	15·8	12·72	11·62
50	13·48	13·36	7·1	14·13	14·15	16·4	12·74	11·12

been due to reaction with the dioxan since after 3 days the pH of the concentrated dioxan solutions had fallen below that of the original sodium hydroxide (Table 1).

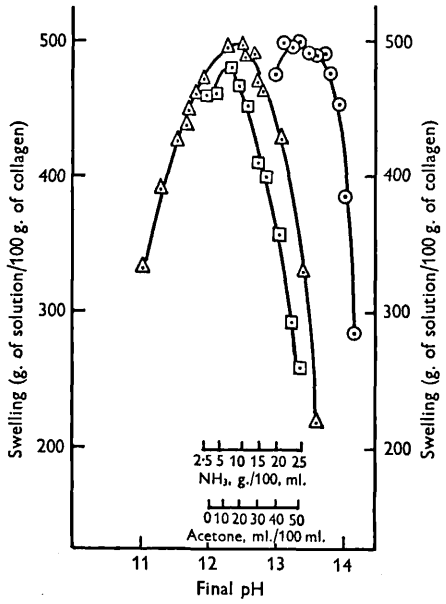

Fig. 5. The effect of increasing additions of acetone on the swelling of collagen in 0·01 (⊡—⊡) and 0·1 M-NaOH (⊙—⊙) solutions. The swelling curve obtained with NH_3 (△—△) is included for comparison.

The swelling of the collagen decreased as the concentration of the acetone increased and in 50 % (v/v) acetone the swelling was of the same order as in the isoelectric range. With acetone in 0·01 N-sodium hydroxide the net result of these two effects, rise in pH and decrease in swelling, was to give a swelling curve similar in shape to those found with the weak bases (Fig. 5) except that it descended more sharply with pH, since there was no increase in hydroxyl ion concentration due to dissociation of base. With 0·1 N-sodium hydroxide the curve was of the same shape,

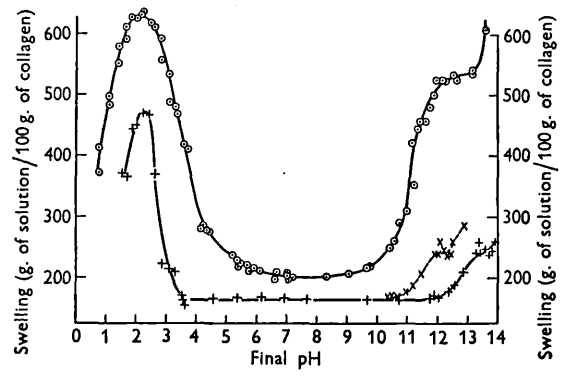

Fig. 6. The effect of the addition of 50 % (v/v) acetone on the swelling of collagen in HCl and NaOH. No acetone, ⊙—⊙; 50 % acetone, +—+, and 50 % acetone, allowance made for effect on K_w, × --- ×.

but was shifted to correspondingly higher pH values. Acetone also decreased swelling in the isoelectric range and in acid solutions (Fig. 6).

The effect of organic solvents on solution of collagen

Although acetone and dioxan decreased swelling in alkaline solutions, they also tended to favour solution of the collagen (Tables 1 and 2). Other

organic solvents miscible with water, e.g. *iso*propanol and pyridine, also had a similar effect (Table 2). If the hydroxyl ion concentration exceeded about 0·5 M the above solvents at 5 M concentrations and 20° caused almost complete solution of the collagen in 3 days.

Table 2. *The effect of organic solvents on the amount of collagen dissolved during 3 days' soaking in sodium hydroxide solutions at 20°*

Solvent	Normality NaOH	pH at 20°	Percentage loss in weight
Dioxan (40%, v/v)	0·79	Approx. 14·1	Nearly all dissolved
	0·53	Approx. 14·1	Nearly all dissolved
	0·26	Approx. 14·1	All dissolved except grain
n-Propanol (37%, v/v)	0·63	13·9	Dissolved in 1 day
	0·30	13·7	Dissolved in 2 days
Pyridine (40%, v/v)	0·10	13·8	80
	0·08	13·7	56
	0·05	13·7	14
	0·04	13·6	8
	0·02	13·4	2
Diethylamine (40%, v/v)	0·002	13·5	2
Acetone (36%, v/v)	0·63	14·1	Dissolved in 1 day
	0·30	14·0	Dissolved in 2 days
Acetone (50%, v/v)	0·05	13·9	3·5
	0·03	13·8	2·2
	0·02	13·7	2·7
	0·015	13·6	2·5
	0·01	13·4	2·4
	0·007	13·3	2·0
	0·002	12·6	0·9

DISCUSSION

The results of this investigation confirm the suggestion that the increased swelling at high pH values in sodium hydroxide solutions is associated with the presence of a high concentration of hydroxyl ions rather than with any specific effect of the sodium ion. The other strong bases studied gave swelling curves of the same shape as those obtained with sodium hydroxide and the addition of 0·5 M-sodium chloride did not alter the shape of the ammonia curve, but reduced swelling at all pH values. Variations in swelling caused by the different bases, both strong and weak, at pH values up to 12·5 can be attributed to variations in the extent to which the cations combine with collagen, thus reducing their contribution to the swelling pressure. On this basis the guanidinium cation has a much greater, and the ammonium ion a slightly greater, affinity for collagen than the cations of the other bases studied. The power of guanidine salts to denature proteins may be related to the high affinity of the guanidinium ions for proteins. The decrease in swelling at high pH values in solutions of weak bases, in apparent agreement with the Donnan theory, is less readily explained. It was thought at first that the presence of the undissociated base in some way inhibited the effect of the hydroxyl ions on cohesion (Bowes & Kenten, 1947), but since depression of the swelling was found to be dependent on the percentage rather than on the molar concentration of the base it seemed more likely that it was related to changes in the medium. The fact that swelling curves of similar shape are obtained by progressively increasing the concentration of acetone in 0·01 and 0·1 N-sodium hydroxide solutions lends support to this view. However, there are also specific differences between the bases, for the extent to which swelling is decreased is not entirely dependent on the percentage composition of their solutions; e.g. swelling in diethylamine solutions is much less than that in solutions of other bases at similar or higher percentage concentrations.

The decrease in swelling with increasing concentration of weak base or organic solvent must be due either to a lowering of the swelling pressure or to an increase in cohesion. Since the presence of organic solvents tended to disperse collagen, which is in itself an indication of decreased cohesion, the former suggestion seems the more likely. Some effect analogous to the 'salting out' of soluble proteins may also play a part as the effects are apparent even when the swelling pressure is very low, e.g. at very high or low pH values and in the isoelectric range.

The mechanism by which the swelling pressure is decreased is not at present clear. It is unlikely, judging from the titration curves of proteins in organic solvents, and in particular of gelatin (Lichtenstein, 1940), that it can be attributed to alterations in the ionization of the reactive groups of the collagen. It is possible that it may be connected with the effect of the solvents on the activities of the ions. The distribution of the ions between the internal and the external phases in the Donnan equilibria is more properly represented by activities than by concentrations. Thus

$$e' = 2a_y y + a_z z - 2a_x x,$$

where e', x, y and z have their usual significance (Bowes & Kenten, 1950; Bolam, 1932) and a_x, a_y and a_z are their respective activity coefficients. In aqueous sodium hydroxide solutions the activity coefficients will not differ greatly from unity, but in the presence of organic substances the activity coefficients will be appreciably lower, and e', and hence the swelling pressure, will be proportionately smaller. To what extent the swelling may be affected by differences between the activity of the components of the solvent phase inside and outside the fibres is not evident.

Although giving no positive evidence regarding the possible reasons for the decrease in cohesion of

the collagen at high pH values, the results are not inconsistent with the view that the main cause is breaking of intermolecular links between the polypeptide molecules. The breaking of hydrogen bonds between polypeptide groups will almost certainly be involved to some extent, but in view of recent work by Lindley (1947), who finds that the specific removal of amide groups greatly increases the solubility of wool in alkaline solutions, links involving these groups may also be concerned. Treatment of collagen in sodium hydroxide solution at pH 13·7 for 3 days decreased the amide nitrogen from 0·36 to 0·12 mmol./g. and caused over 700 % swelling, whereas treatment in ammonia or diethylamine solutions at the same observed pH values had no appreciable effect on the amide nitrogen and caused no swelling. In this connexion it is of interest that Ames (1944) states that loss of amide nitrogen is essential for the production of gelatin from collagen. Another possibility is that the decrease in cohesion is related to removal of mucopolysaccharides. Partridge (1948), as a result of work on the state of combination of chondroitin sulphate in cartilage, suggests that the chondroitin sulphate, acting as a multivalent anion, cements together the protein molecules to form fibrous macromolecules. He finds that breakdown of the gelatin-chondroitin sulphate complex causes a large decrease in the viscosity of gelatin. Treatment with alkali or calcium chloride are common methods of extracting chondroitin sulphate from cartilage and other tissues, and since both these treatments lead to a decrease in cohesion (for information on the effect of calcium chloride see Bowes, 1950) it seems possible that its removal may at least be a contributory cause of this decrease.

SUMMARY

1. The swelling of ox-hide collagen in solutions of strong and weak bases has been studied. Differences in swelling at pH values up to pH 12·5 are ascribed to variations in the affinity of the cations for the collagen.

2. The swelling curves obtained with tetramethylammonium hydroxide and guanidine were of the same general shape as the curve obtained with sodium hydroxide, suggesting that the increased swelling at high pH values is due to increase in the hydroxyl ion concentration rather than to any specific effect of the sodium ion.

3. With weak bases swelling decreased at pH values above 12·5, in apparent accordance with the Donnan theory. Curves of similar shape were obtained by the addition of increasing amounts of neutral organic solvent, and it is concluded that the decrease in swelling is due to the progressive departure from an aqueous medium. There were also specific effects due to the different bases.

4. The effect of the organic solvents was presumably due to decrease in the swelling pressure rather than to increase in cohesion, since their presence was found to favour solution of the collagen.

5. The results are consistent with the view that decrease in the cohesion of collagen at high pH values is due to breaking of intermolecular linkages. The possible nature of these linkages is discussed.

The authors wish to thank Prof. C. W. Davies and Dr D. H. Everett for advice on the interpretation of pH measurements in solutions of weak bases and organic solvents, and the Council of The British Leather Manufacturers' Research Association for permission to publish this paper.

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[FROM THE BIOCHEMICAL JOURNAL, VOL. 46, No. 5, pp. 530–532, 1950]

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The Swelling of Collagen in Alkaline Solutions

3. SWELLING IN SOLUTIONS OF BIVALENT BASES

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(*Received* 22 *September* 1949)

The swelling of collagen in solutions of a number of univalent bases has been described in previous communications (Bowes & Kenten, 1950*a*, *b*). In the present paper the work has been extended to cover the bivalent bases, calcium, strontium and barium hydroxide.

EXPERIMENTAL

The raw material and technique were the same as those used in Parts 1 and 2 (Bowes & Kenten, 1950*a*, *b*). Saturated solutions of $Ca(OH)_2$, $Sr(OH)_2$ and $Ba(OH)_2$ were prepared by shaking excess of the solid hydroxide with boiled distilled water and allowing to settle.

RESULTS

Swelling in solutions of calcium, strontium and barium hydroxides

The swelling of collagen in solutions of calcium, strontium and barium hydroxides was considerably less than in sodium hydroxide solutions at all pH values, and more especially in the range 11·0–13·0 (Fig. 1). The curves were of the same general shape as the sodium hydroxide curve, showing a flattening between pH 12·0 and 13·0 followed by a sharp increase in swelling at higher pH values. Appreciable swelling commenced only at about pH 11, i.e. approximately one pH unit higher than with sodium hydroxide. The amount of swelling decreased in the order barium, strontium, calcium hydroxide. Even in barium hydroxide solutions the swelling, in excess of that occurring in the isoelectric range, was barely half that in sodium hydroxide solutions, that in strontium hydroxide was rather less and that in calcium hydroxide was only about one-eighth of that in sodium hydroxide solutions.

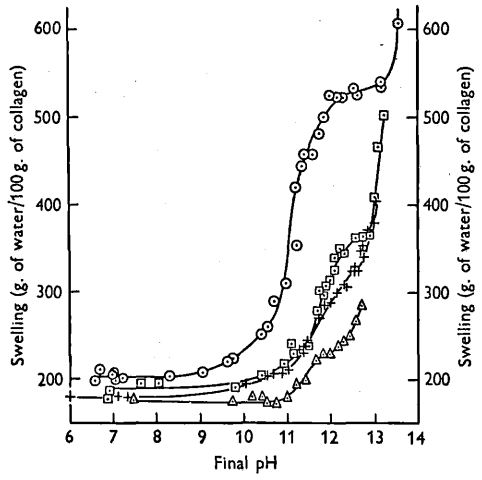

Fig. 1. Swelling of collagen in solutions of $Ca(OH)_2$, △—△; $Sr(OH)_2$, +—+; $Ba(OH)_2$, ⊡—⊡. Curve ⊙—⊙ for NaOH solutions included for comparison.

The effect of calcium and sodium chlorides on swelling in calcium hydroxide solutions

The addition of calcium chloride increased the swelling in calcium hydroxide solutions at all pH values (Fig. 2), the presence of 0·09 M-calcium chloride slightly increased the swelling, and as the concentration was further increased the swelling progressively increased until in the presence of 1·0 M-calcium chloride it was more than twice that in the hydroxide alone. The addition of a salt must decrease the swelling pressure, and the fact that calcium chloride increased swelling indicates that it must have a very marked effect in decreasing the cohesion of the collagen.

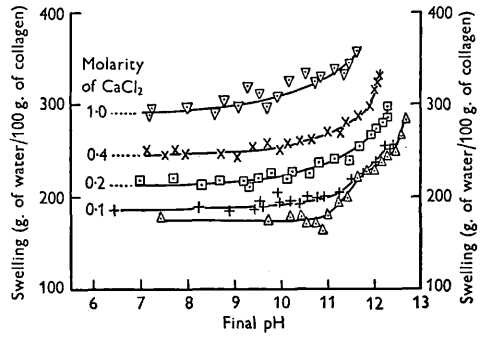

Fig. 2. The effect of addition of $CaCl_2$ on the swelling of collagen in $Ca(OH)_2$ solutions (△—△, no $CaCl_2$).

The addition of sodium chloride also increased swelling in calcium hydroxide solutions (Figs. 3 and 4). As low a concentration as 0·001 M-sodium chloride

caused a small increase in swelling, and with 0·02 M-sodium chloride the swelling was approximately doubled. The swelling continued to increase until the concentration reached 0·1 M and then remained approximately constant as the concentration was further increased to 0·2, 0·5 and 1 M. Above 0·1 M

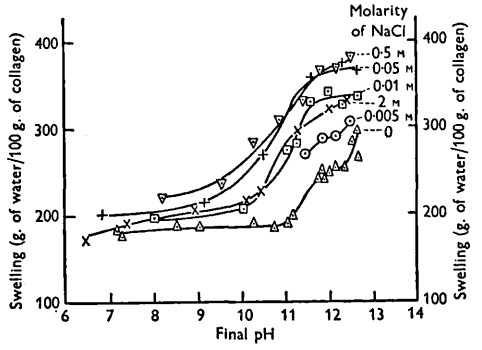

Fig. 3. The effect of addition of NaCl on the swelling of collagen in $Ca(OH)_2$ solutions.

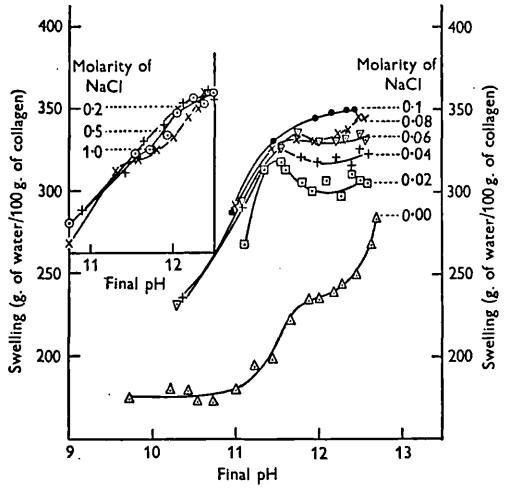

Fig. 4. The effect of addition of NaCl on the swelling of collagen in $Ca(OH)_2$ solutions.

the shape of the curve began to change, the flattening in the pH range 12·0–12·5 was less marked and the curve tended to descend more sharply with decrease in pH until it became concave in shape. Similar changes in the shape of the swelling curves in sodium hydroxide solutions with increasing sodium chloride concentration have already been described (Bowes & Kenten, 1950*a*).

The maximum shown in the neighbourhood of pH 11·5 with 0·02 and 0·04 M-sodium chloride may be due to changes in the calcium-ion concentration. A change in pH from 11·5 to 12·5 involves a tenfold change in the calcium-ion concentration, and since the sodium-ion concentration is constant and low, it is possible that such a rise may increase the Ca^{++}/Na^{+} ratio sufficiently to cause a decrease in the swelling.

DISCUSSION

On the basis of the Donnan theory of membrane equilibria it might be expected that the swelling pressure and hence the amount of swelling would be the same with all three alkaline earth bases, and rather more than half that in sodium hydroxide solutions at corresponding pH values, the ratio of the membrane potentials in uni- and bi-valent systems being 0·66 (Bolam, 1932). Only with barium hydroxide, however, did the swelling approach half that found with sodium hydroxide and with calcium hydroxide it was very much less. The low amount of swelling in calcium hydroxide is the more surprising when it is remembered that the second dissociation constant of calcium hydroxide is comparatively low, 0·031 (Davies, 1938), so that about one-third of the calcium functions as a monovalent cation, whereas with barium hydroxide the corresponding dissociation constant is 0·23 (Davies, 1939) and nearly all the barium will be present as the divalent cation.

The unexpectedly low swelling in solutions of bivalent bases, especially calcium hydroxide, may be due to their having a smaller effect on the cohesion than sodium hydroxide, or to incomplete ionization of the protein salts. In view of the relative effects of calcium and sodium chlorides on swelling in calcium hydroxide (Fig. 2), and in sodium hydroxide (Bowes & Kenten, 1950*a*), respectively, and of the lowering of the shrinkage temperature caused by calcium chloride (Theis & Steinhardt, 1942), it would appear that the calcium ion reduces the cohesion of the protein to a much greater extent than the sodium ion and the first suggestion is, therefore, improbable. There is a considerable amount of evidence pointing to the combination of alkaline earth cations, especially calcium, with proteins (Greenberg, 1944) and by analogy with the dissociation constants of calcium and barium salts of the hydroxy and dicarboxylic acids (Cannan & Kibrick, 1938), calcium is likely to have a greater affinity than barium for proteins. The present observations suggest that the affinity of calcium, strontium and barium for collagen decreases in the order $Ca > Sr \geqslant Ba$. No evidence is at present available regarding the relative effect of these three cations on cohesion.

It seems that three factors participate in determining the effect of additions of sodium chloride to solutions of calcium hydroxide (or of any univalent salt to a solution of a bivalent base) on the swelling of collagen: (1) The effect of the univalent cation in increasing the swelling pressure. (2) The effect of

the salt in decreasing the swelling pressure. (3) The effect of the salt on cohesion. The curves shown in Fig. 3 suggest that at low concentrations of sodium chloride, increase in the sodium-ion concentration is the predominant factor, the rise in the Na^+/Ca^{++} ratio increasing the swelling pressure with the result that swelling increases. As the concentration of sodium chloride increases, the effect of salt in depressing the swelling pressure becomes apparent, and balances the effect of increase in the sodium-ion concentration, so that the swelling remains constant. The change in the shape of the curve above 0·1 M suggests that cohesion is beginning to be affected; at concentrations of this order the swelling pressure probably begins to decrease, but the decrease in cohesion balances this and the swelling remains unchanged. At concentrations of 2 M or greater, sodium chloride begins to decrease the swelling again, probably by an action similar to that occurring in the salting out of proteins (Cohn & Edsall, 1943).

SUMMARY

1. The swelling of collagen in solutions of bivalent bases was found to decrease in the order barium hydroxide ≥ strontium hydroxide > calcium hydroxide. Swelling in barium hydroxide solutions was rather less than half, and that in calcium hydroxide solutions about one-eighth of that in sodium hydroxide solutions. The small amount of swelling in calcium hydroxide solutions is attributed to combination of calcium ions with the collagen.

2. The addition of calcium or sodium chlorides increased swelling in calcium hydroxide solutions. The increase with calcium chloride is attributed to decrease in the cohesion of the collagen, and the increase with sodium chloride to the addition of a univalent cation and consequent increase in swelling pressure.

The author thanks the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

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RECENT STUDIES OF THE CHEMISTRY OF THE LIMING PROCESS.

By J. H. Bowes.

Reprinted from Journal Society of Leather Trades' Chemists, 1949, **33**, 176.

RECENT STUDIES OF THE CHEMISTRY OF THE LIMING PROCESS.

By J. H. Bowes.

A number of factors are involved in liming : (1) the removal of hair or wool ; (2) chemical action of the alkali on the skin proteins, collagen, elastin, and reticular tissue ; (3) physical action on the collagen and other proteins, such as swelling, and removal of constrictive forces holding the collagen fibres together.

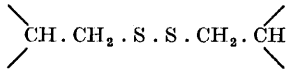
Removal of the hair or wool is essential for most types of leather, and the efficiency with which this process is carried out has an important influence on the appearance of the leather. Chemical action on the skin collagen may affect its behaviour in the subsequent processes and its capacity to combine with tanning agents, and physical actions such as swelling and opening up will have important effects on the physical properties of the resulting leather.

It is proposed to discuss present knowledge of the chemistry of the liming process, dealing more especially with work recently carried out in these Laboratories.

Removal of Hair.

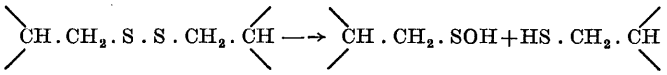
The removal of the hair or wool is dependent on the breakdown of the disulphide link of the amino acid cystine in the keratin molecules, and perhaps also to effects on the cementing substances holding the hair root in the follicle.

The disulphide link forms a cross link between adjacent polypeptide chains:



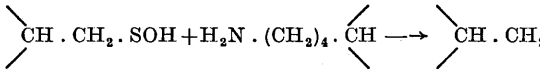
When the disulphide link is broken the stability of the keratin is decreased, the hair is weakened and finally disintegrates. The hair root and the base of the hair shaft are more susceptible to attack, than the main shaft[1], and hence it is possible to loosen the hair without causing appreciable damage to the bulk of it.

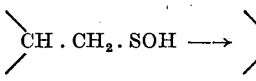
It has been suggested by various workers[2, 3, 4, 5, 6] that the disulphide link is hydrolysed in alkaline solution to give sulphenic acid and thiol groups :

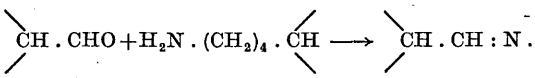


In alkali alone, however, new cross links can be formed which restabilise the structure. This is the reason why alkaline solutions alone will not readily unhair, and why difficulty is sometines experienced in unhairing hides or skins which have been soaked in alkaline solutions[1, 8, 9].

It was first suggested that cross links involving the amino groups were formed as shown in equations 1 and 2:

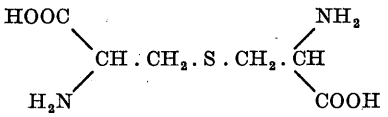
(1)  [2, 6]

(2)  [4, 5]

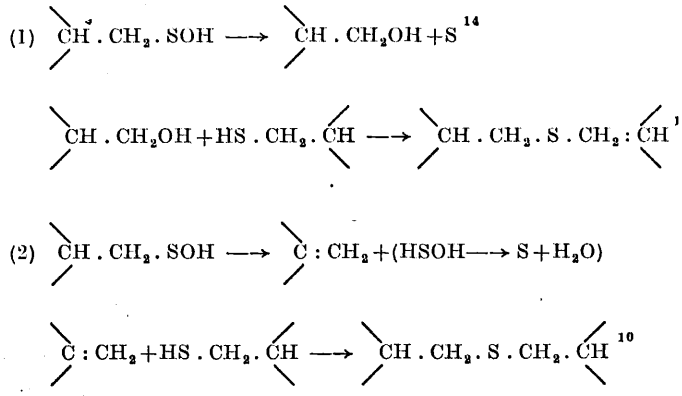
 [9]

The formation of such cross links should leave free thiol groups in the wool, and failure to demonstrate the presence of these[5, 10], or of aldehyde groups, or to find any decrease in the amino-nitrogen[10] suggests that they cannot occur to any great extent.

It was suggested by Speakman and Whewell[11] in 1936 that thioether cross linkages were formed, and their existence has now been confirmed by the isolation of lanthionine from alkali-treated wool, first by Horn, Jones and Ringel[12] in 1941, and subsequently by a number of other workers[10, 13] :



The following mechanisms have been suggested for its formation :



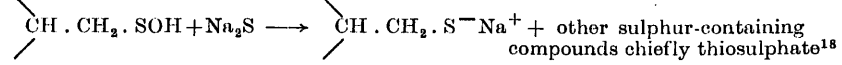
The first mechanism was put forward as a suggestion, and there is no direct evidence in support of it. Cuthbertson and Phillips[10], however, did a considerable amount of work to establish their theory. They found that about half the cystine in wool could be converted into lanthionine by the action of alkalis, while the other half was converted to two molecules of combined amino acrylic acid. It is assumed that the second molecule of amino acrylic acid is formed by the loss of hydrogen sulphide from the thiol group as postulated by Nicolet[16]. Both reactions are favoured by increase in pH, but that leading to the formation of two molecules of amino acylic acid requires a higher pH than that due to the formation of lanthionine. It is suggested that the different reactivity of the two fractions, termed (A+B) and (C+D) by Phillips, is due to their environment. The reactions of (A+B) are probably more rigidly controlled by structural factors and hence reaction between the products of the hydrolysis of the disulphide link readily take place, thus leading to the formation of lanthionine, whereas fraction (C+D) is less restricted, and there is less possibility of such reactions occurring.

Conversion to lanthionine was found to take place more rapidly with barium hydroxide than with sodium hydroxide[10]; 0.38*N* barium hydroxide converted half the disulphide links into lanthionine in three-quarters of an hour at room temperature. By analogy, it may be expected that pre-treatment in solutions of lime will lead to more rapid formation of new cross links, and hence to greater difficulties in subsequent unhairing, than pre-treatment in sodium hydroxide solutions under corresponding conditions. In this connection Anderson[17] has shown that hair is more rapidly made resistant to supercontraction in boiling bisulphite by calcium hydroxide, than by sodium hydroxide solutions, at the same concentration.

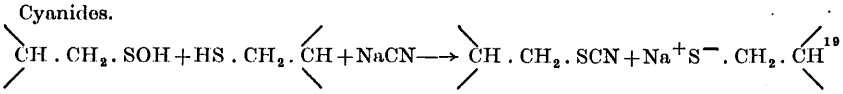
It is the function of the unhairing agent to prevent the formation of new cross links by reacting with the groups involved. Sulphides can act by reducing –SOH groups to SH groups and so preventing the formation of

anthionine—e.g. (1) below, and hydrosulphides and other reducing agents can act in the same way :

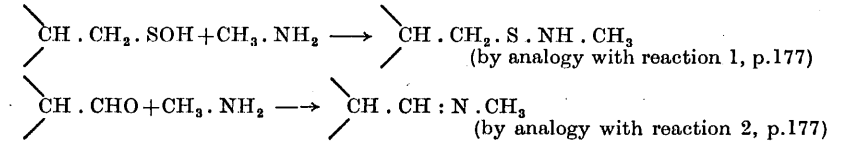
(1) Sulphides and hydrosulphides.



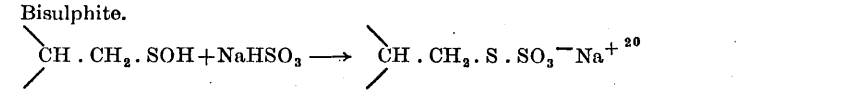
(2) Cyanides.



(3) Amines.



(4) Bisulphite.



Cyanides may have effects other than that shown in equation 2, since Cuthbertson and Phillips[10] have recently found that in neutral solution potassium cyanide converts all the cystine in wool to lanthionine. It has been suggested that amines react as shown in equations 3 above, so preventing the formation of cross links involving amino groups, but since the existence of such links now appears doubtful, some other mechanism will have to be sought. It is possible that –S.NH– links are not formed in the protein owing to the position of the –SOH and $-NH_2$ groups relative to one another, but that the amines can react with –SOH groups as shown above, and so prevent the formation of lanthionine.

To summarise, therefore, unhairing is dependent on two reactions, hydrolysis of the disulphide link, and the reaction of the unhairing agent with the products of this hydrolysis, or other groups involved in the formation of new cross-links. The first reaction is dependent on the hydroxyl ion concentration, and the second on the concentration of the unhairing agent; the higher the concentration of either of these, the greater the action on the hair.

The effect of the hydroxyl ion concentration on unhairing is illustrated by the results in Table I. Pieces of goatskin were placed in a series of solutions of constant sulphide concentration, but of varying hydroxyl ion concentration, and the ease with which the hair could be removed tested at intervals. The extent to which the hair was loosened increased with hydroxyl ion concentration ; at the lower values it was still tight at the end of six days, whilst at the highest concentrations it could be readily removed after 48 hr. There was a fairly sharp increase in the rate of unhairing between concentrations of 17 and 36 mg. equiv. per litre, unhairing being complete in half the time at the higher concentration. It was also noted that a much cleaner grain was obtained when the hydroxyl ion concentration was high.

The question of the mechanical removal of the hair as well as the chemical action of the liquor on the hair must be considered in unhairing. The hair root is the part most vulnerable to attack by sulphides and other unhairing agents[1]. If the skin is swollen the neck of the hair follicle is constricted, and although the hair root may be loosened, it cannot readily be pulled out, more drastic chemical action is, therefore, required in order to pulp the hair root,

TABLE I.

THE EFFECT OF (OH^-) ON UNHAIRING.

EXPERIMENT I.				
pH at 20°C	11·44	11·77	12·45	12·89
(OH^-) mg. eq. per litre ...	2	4	19	52
UNHAIRING AFTER.				
24 hours	+	+	++	++++
48 „	+	++	+++	+++++
56 „	++	+++	++++	+++++
80 „	++	+++	+++++	+++++
EXPERIMENT II.				
pH at 20°C	11·85	12·11	12·40	12·72
(OH^-) mg. eq. per litre ...	5	9	17	36
UNHAIRING AFTER.				
20 hours	++	++	++	+++
44 „	+++	+++	+++	++++
72 „	+++	++++	++++	+++++
96 „	+++	++++	+++++	+++++

and so allow it to be removed. Under such conditions the hair may be weakened at the surface, and under the action of the unhairing machine may break off, leaving the degraded hair root in the skin. The optimum conditions for removal of the hair roots are, therefore, a mild action on the hair combined with the minimum amount of swelling.

Chemical Action on the Collagen.

In order that the hair may be removed in a reasonable time, comparatively high concentrations of alkali are necessary, and under such conditions the collagen may be affected. Not only does the collagen combine with alkali, but modification of certain of the reactive groups may take place.

It has been suggested at various times that the following changes may take place :—

(1) Hydrolysis of amide groups with the production of ammonia[7, 21, 22, 23];

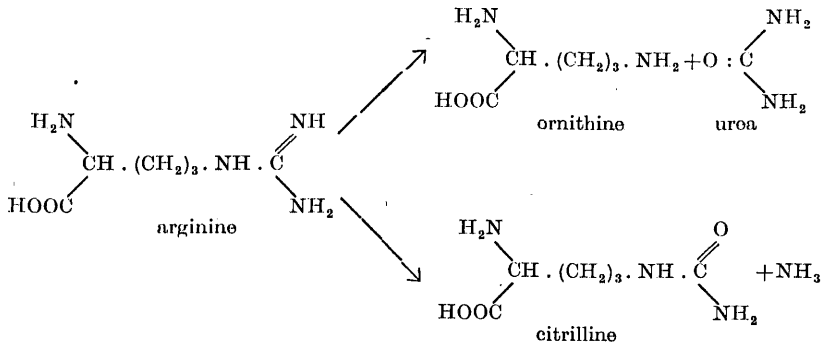
(2) Modification of guanidino groups[22, 24];

(3) Destruction of the hydroxy amino acids serine and threonine[25];

(4) Hydrolysis of peptide groups of the main polypeptide chains.

Marriott[21] has demonstrated loss of amide groups when collagen is treated with alkali, and he[1, 7, 21] and numerous workers[22, 23, 26, 27] report the production of ammonia.

Hellermann and Stock[28], and Warner[29], have shown that the following reactions take place when arginine is treated with alkali and it is possible that similar reactions occur with the arginine residues in the intact protein :



In support of this, Highberger and Stecker[22] have shown that urea is formed when collagen is treated with alkali.

Nicolet, Skinn and Saidle[30] have shown that treatment in boiling decinormal alkali for 1–2 hr. causes considerable destruction of the hydroxyamino acids in silk, and it is possible that a similar reaction may occur to some extent in the cold over long periods of time.

There is little information regarding the extent to which hydrolysis of peptide links occurs under the conditions of liming. When the concentration of alkali is high, appreciable amounts of collagen may be dissolved, but under the conditions usually met with in practice this is probably very small.

Thus, although a considerable amount of work has been done on the effect of alkalis on collagen, the evidence is on the whole, qualitative rather than quantitative. Further each worker has dealt with only one or two of the possible changes which may occur, and only in one instance has a collagen been used which has had no previous alkaline or enzyme treatment[21]. It was considered desirable, therefore, to carry out an experiment in which both the changes in the protein and the soluble decomposition products were considered in some detail[31]. Ox hide collagen, which had received no previous chemical treatment, other than washing with 10% sodium chloride to remove interfibrillary proteins and degreasing in llght petroleum, was treated in a solution of calcium and sodium hydroxides (initial pH 13.3 and final pH 13.0 at 20°C.) for 14 days at 20°C., and the treated collagen and the solution analysed. (See Table II.) Some of the data given was obtained direct from analysis, while some was deduced from consideration of the titration curves of the collagen before and after treatment.

The chief reaction occurring was hydrolysis of amide groups with production of an equivalent amount of ammonia. About half the amide groups were hydrolysed, but on longer treatment under the same conditions a further 0.08 millimoles per g. were lost, and it seems probable that all the amide groups would be lost if the treatment were sufficiently prolonged. In an independent experiment it was found that the amide nitrogen of sheepskin collagen was reduced from 0.35 to 0.22 and 0.12 millimoles per g. respectively by treatment for three days at pH 13.4 and 13.7. Thus hydrolysis of amide groups increases with pH and time of treatment, a conclusion essentially the same as that reached by Highberger and Stecker from consideration of the ammonia produced.

A small amount of arginine was lost during the treatment and an approximately equivalent amount of urea was found in the solution. Thus it seems

TABLE II.

ANALYSIS OF ALKALI-TREATED COLLAGEN.

		Collagen			
		Untreated	Alkali treated	Solution	
FROM ANALYSIS :					
Total–N %		18·6	18·2	Total–N mg./g. ...	9·0
Amide–N... ...	mmols./g.	0·47	0·22	Ammonia–N mmols./g.	0·25
Amino–N ...	„	0·33	0·36	Amino–N mg./g. ...	4·3
Arginine	„	0·48	0·46	Urea mmols./g. ...	0·02
Periodate–NH_3 ...	„	0·60	0·58		
FROM TITRATION CURVE :					
Total basic groups	„	0·90	0·96		
Dicarboxylic acids	„	1·32	1·26		
α amino + imino	„	0·02	0·08		

probable that the reaction leading to the formation of amino groups predominates over that leading to the production of citrulline residues. The treated collagen gave a faintly positive test for citrulline by Fearon's test[32], whereas the original collagen did not, indicating that a small fraction of the arginine had been converted to citrulline. There was no significant decrease in the hydroxyamino acid content of the treated collagen.

There was a small increase in the free amino-nitrogen of the collagen from 0.33 to 0.36 millimoles per g. and a larger increase, 0.06 millimoles per g., in the total number of basic groups. The increase in free amino-groups can largely be accounted for by the production of ornithine from arginine, and there can be little, if any, hydrolysis of peptide groups involving amino groups. It is probable that the increase in total basic groups is due to hydrolysis of peptide groups involving the imino group of proline or hydroxyproline; if this is so, it suggests that such peptide links are more susceptible to the action of alkali than those involving amino groups.

Although there is only a small increase in the number of terminal basic groups in the alkali-treated collagen, about 5% of the collagen was dissolved, and the amount of nitrogen in solution was considerably in excess of that due to the ammonia and urea. The high value for the amino-nitrogen of the solution suggests that this nitrogen was present as amino acids or small peptides. The lower dicarboxylic acid content of the treated collagen compared with that of the original collagen suggests that the fraction dissolved may be rich in the dicarboxylic acids.

The total nitrogen, amide-nitrogen and amino-nitrogen of a number of commercially limed pelts were determined, to see how these quantities varied in practice[31]. (Table III.) The total nitrogen content of all the samples was lower than that of the untreated ox hide collagen, that of the limed ox hides being about 18.1% and that of the sheepskins about 17.3%. The lower value for the sheepskins cannot be accounted for by loss of nitrogen from amide or guanidino groups, and suggests that their original composition may be different from that of ox hides. In this connection it may be noted that the factor 5.62

used in the conversion of nitrogen to hide substance in leather analysis corresponds to a nitrogen content of 17.7%, and hence in general does not give an exact figure for the collagen content[33]. The amide-nitrogen values of the ox hides and calf skins indicates that there has been little loss during liming. The values for the sheep skins are somewhat lower ; this may be due to greater loss during processing or possibly to a lower original content. The values for the amino-nitrogen are little greater, and in some cases less, than that of the original untreated ox hide collagen and indicate that there can be little breaking of peptide links during liming.

TABLE III.

TOTAL NITROGEN, AMIDE-NITROGEN AND AMINO-NITROGEN OF SOME COMMERCIALLY LIMED PELTS.

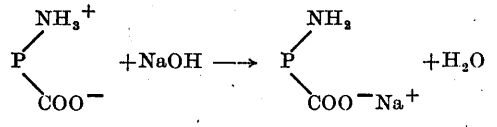
(All results expressed on Moisture and Ash-free Collagen)

Pelt		Total Nitrogen %	Amide-Nitrogen %	Amide-Nitrogen mmols./g.	Amino-Nitrogen %	Amino-Nitrogen mmols./g.
Ox Hide Collagen— no Alkaline Treatment		18·6	0·66	0·47	0·46	0·33
Sheep Skins	A	17·3	0·42	0·30	—	—
	B	17·1	0·42	0·30	0·59	0·42
	C	17·2	0·35	0·25	0·50	0·36
	D	17·4	0·50	0·36	0·45	0·32
	E	17·3	0·49	0·35	0·49	0·35
Calf Skins	1	18·2	0·56	0·40	—	—
	2	18·2	0·52	0·37	—	—
	3	18·0	0·55	0·39	0·42	0·30
Ox Hide	1	18·1	0·66	0·47	0·46	0·33
	2	18·1	0·42	0·30	0·45	0·32
	3	18·3	0·46	0·33	0·48	0·34
	4	18·1	0·57	0·41	0·42	0·30

From the above discussion it may be concluded that chemical modification of the collagen during liming is comparatively small, hydrolysis of amide groups being the only reaction occurring to an appreciable extent, but that at pH values above 13.0 appreciable amounts of collagen may be dissolved. The physical changes taking place are probably of greater importance. Comparison of the swelling curves of the original and the alkali-treated collagen (Fig. 1) shows that the alkaline treatment has appreciably increased the water uptake at all pH values. These differences cannot be accounted for by changes in the reactive groups, but must be due to physical changes in the collagen.

The Swelling of Collagen in Alkaline Solutions.

When collagen is placed in alkaline solutions it reacts with hydroxyl ions :



It then carries a negative charge, and because of this cations, in the above case sodium ions, become associated with it ; this leads to a higher

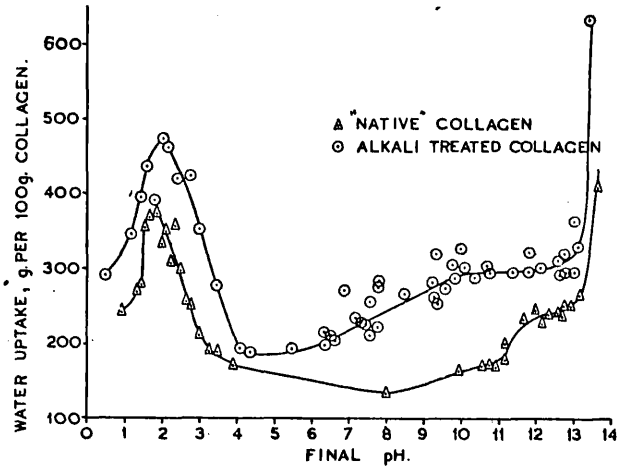


FIG. 1.

Water Uptake of " Native " and Alkali-treated Collagen.

concentration of sodium ions inside the protein fibre than in the external solution, and owing to the Donnan effect, what may be termed a swelling pressure is set up, water passes into the fibre in order to equalise the concentration, and the fibre swells. (For a full discussion of the Donnan Theory as applied to the swelling of proteins, see Bolam[34] and Wilson [35].)

The amount of water taken up depends not only on the swelling pressure but on the forces opposing this pressure and the entry of water into the collagen. These may be referred to as the cohesion of the collagen, and include structural features such as the interweaving of the fibres, reticular sheaths round the fibres, and chemical forces holding the long polypeptide chains together. The importance of structural features and the mechanical effect of the interweaving of the fibres in restricting swelling is shown by comparing the amount of water taken up by ox hide, and sheep skin collagen under the same conditions. (Fig. 2.) Single ox hide fibres, which are free from any restrictions due to interweaving, would take up considerably more water; at pH2 the water uptake of such fibres would be of the order of 2000%.

According to the Donnan theory, the water uptake should increase as the pH rises or falls from the isoelectric point, reaching a maximum at about pH 12 and 2, and then decreasing again as the pH is further increased or decreased. In hydrochloric acid solutions this in fact does take place. (Fig. 3.) On the alkaline side of the isoelectric point, however, the water uptake increases from the isoelectric point to about 11.5, increases less rapidly between pH 11.5 and 13.0, and then increases very sharply with further rise in pH[36]. This increase in water uptake at high pH values cannot be explained on the basis of liberation of new groups which combine with alkali, and from consideration of the curve in relation to the Donnan theory of membrane equilibria it can be shown that the swelling pressure must start to decrease at pH values above about 12. It may be concluded, therefore, that the increase in water uptake at high pH

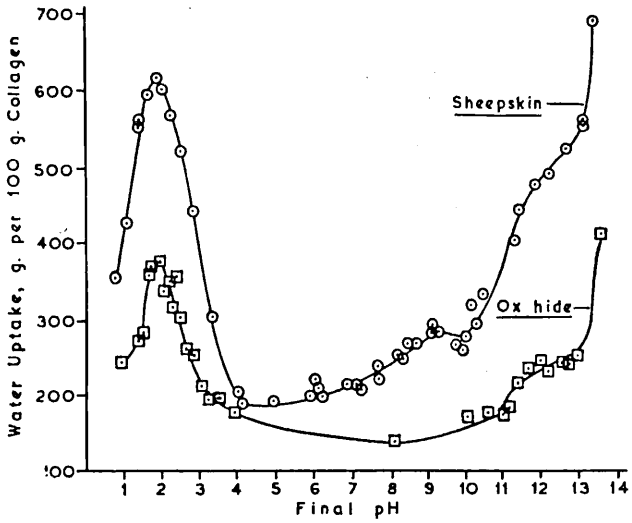


FIG. 2.
Water Uptake of Ox Hide and Sheep Skin Collagen.

values is due to a decrease in the cohesion of the collagen, so allowing it to take up more water. Calculation also shows that the uptake of water at pH 12 is greater than would be expected from the amount of base bound, so that some decrease in cohesion occurs even at this pH value. A study of the water uptake with other strong bases indicates that this effect on cohesion is due to the hydroxyl ion and not to any specific effect of the sodium ion.[37].

The addition of sodium chloride does not decrease the water uptake in alkaline solutions to the same extent as in acid solutions ; 0.2M sodium chloride decreases water uptake in accordance with theory, but higher concentrations have little further effect. (Fig. 3.) It is concluded that sodium chloride also decreases cohesion and other salts will presumably have a similar effect.

The water uptake from solutions of calcium hydroxide is much less than from sodium hydroxide solutions[36]. (Fig. 4.) Since the calcium ion is divalent there will only be half as many calcium ions as sodium ions associated with the collagen, and consequently the swelling pressure should only be about half that in sodium hydroxide solutions of the same pH value. The water uptake, however, is found to be much less than corresponds to this lower swelling pressure. There is evidence that calcium combines with proteins[38] ; if this is the case with collagen, then those calcium ions which are combined will no longer be able to play a part in developing a swelling pressure, so accounting for the low water uptake. The addition of calcium chloride to calcium hydroxide solutions does not decrease the water uptake, but progressively increases it as the concentration increases (Fig. 4). The addition of a salt, by decreasing the difference in concentration between the protein phase and the external solution, must decrease the swelling pressure, hence the increase of water uptake due to the addition of calcium chloride must be ascribed to decrease in cohesion. The calcium ion appears to have a comparatively large effect in this respect.

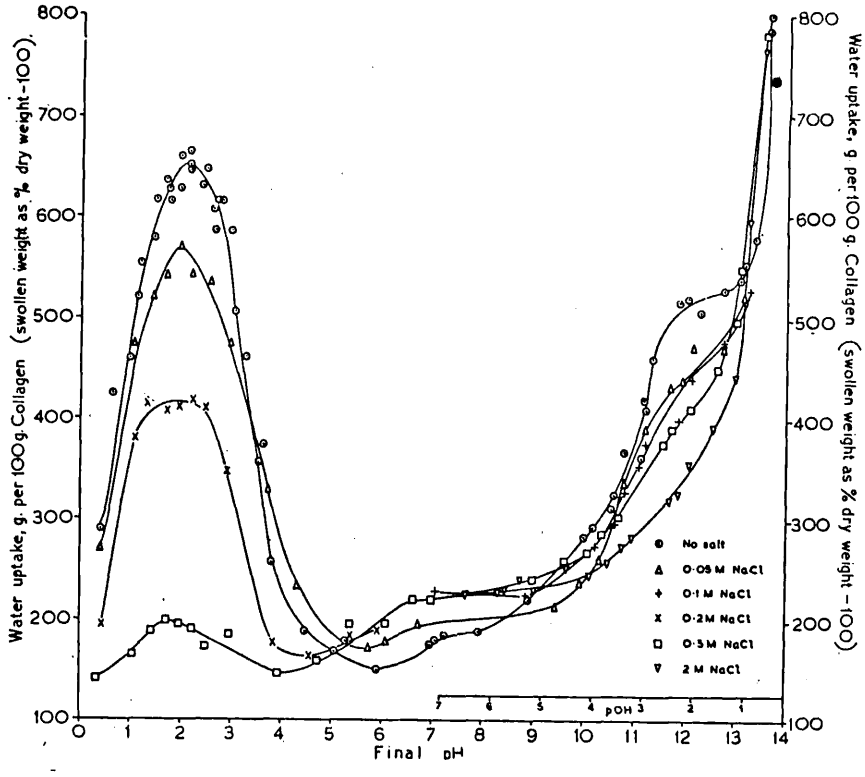


FIG. 3.

Water Uptake of Collagen in Hydrochloric Acid/Sodium Chloride and Sodium Hydroxide/Sodium Chloride Systems at 20°C.

It is interesting to consider the effect on swelling of the addition of sodium chloride to a solution of calcium hydroxide[36]. The addition of the monovalent sodium ion will tend to increase the swelling pressure, the addition of the salt will tend to decrease it, and at concentrations above $0.2M$ effects on cohesion may be expected. Experimentally the swelling curves shown in Fig. 5 are obtained. The water uptake increases as the sodium chloride concentration increases from 0 to $0.05M$ (0.3%), remains approximately constant as the concentration is further increased to $0.5M$ (3%) and at still higher concentrations ($2M$) decreases again.

These results may be interpreted as follows :—At low concentrations the effect of the sodium ion on the swelling pressure is predominant, and the swelling pressure increases and reaches its maximum value somewhere about $0.05M$; the effect of the salt in decreasing swelling pressure then becomes apparent and the two effects, that of the sodium ion in increasing swelling and that of the salt in decreasing it, balance one another and the water uptake remains constant. At concentrations above $0.2M$ (1.2%) some effect on cohesion may also be expected and this will also help to balance the effect of the salt in decreasing the swelling pressure and so keep the water uptake constant. Other sodium salts will have the same effect on water uptake as

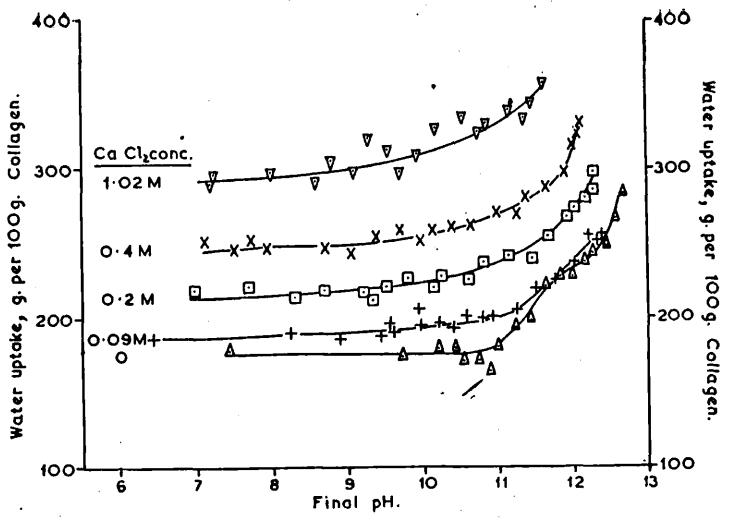


FIG. 4.

Water Uptake of Collagen from Calcium Hydroxide Solutions with and without the addition of Calcium Chloride.

sodium chloride at equivalent concentrations, but may in addition have other effects, such as increasing the (OH^-) as, for example, sodium sulphide.

Although the water uptake is the same over a wide range of salt concentration, the effect on the collagen is not necessarily the same; at low salt concentrations the water uptake is mainly due to an increase in swelling pressure while at high concentrations it is mainly due to a decrease in cohesion.

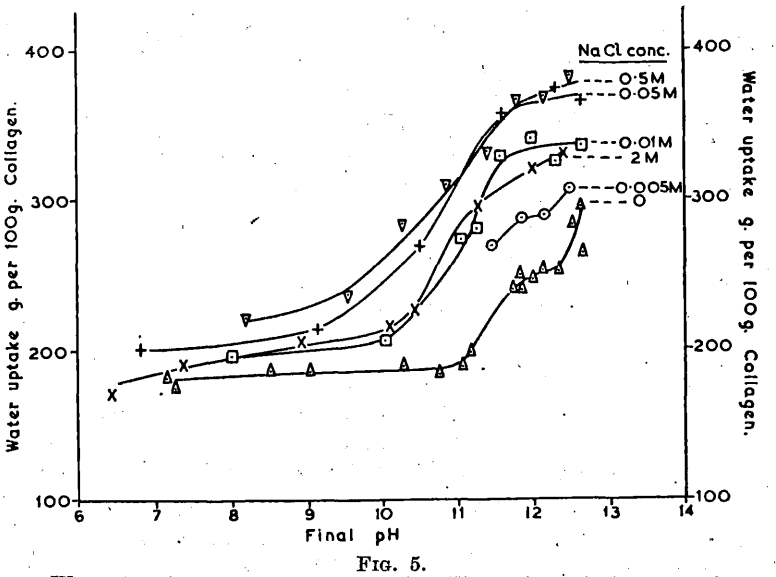


FIG. 5.

Water Uptake of Collagen from Calcium Hydroxide Solutions Containing Sodium Chloride.

The decrease in cohesion occurring in alkaline solutions may be due to alteration in structural features such as the breaking of reticular or other sheaths around the fibres and fibre bundles, loosening of the fibre weave, etc., or to the breaking of intermolecular forces holding the polypeptide chains together. Comparison of the swelling curves of collagen and gelatin[36] suggests that both factors play a part, but that at high pH values breaking of intermolecular links is the predominant cause of the decrease in cohesion.

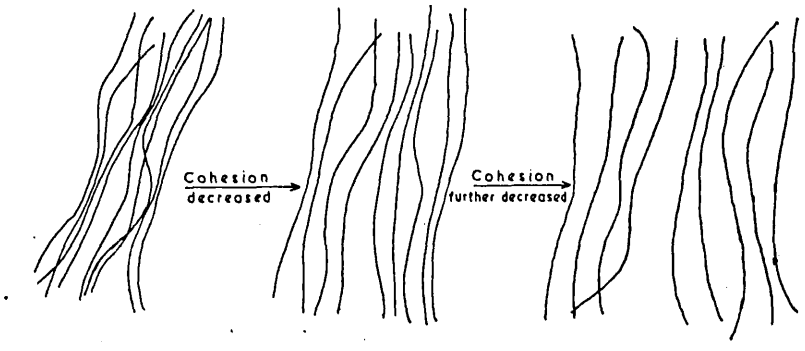


FIG. 6.
Diagramatic Representation of the Decrease in Cohesion of Collagen Fibres in Alkaline Solutions.

Representing the long polypeptide chains from which collagen is built up as shown in Fig. 6, and assuming that they are held together by intermolecular forces along the chain, one can imagine that the breaking of these bonds would allow more swelling to take place and that if this swelling is extensive it may lead to permanent alterations in the molecular arrangement. If the decrease in cohesion is small it is reasonable to believe that on removal of the water the polypeptide chains will come back more or less to their original positions. If the decrease in cohesion is great, however, the chains will become permanently displaced, and in the extreme case will separate altogether, and the collagen go into solution as gelatin. The greater swelling of the alkali-treated collagen compared with that of the original untreated collagen (Fig. 1) shows that the alkaline treatment has caused some permanent alteration in the cohesion of the collagen.

Conclusion.

In addition to the removal of the hair or wool, liming involves both chemical and physical action on the collagen. The amount of chemical modification which will occur under the usual conditions of liming is small, and the changes leading to physical modification of the collagen are probably the more important.

The chief factors determining the extent to which the above effects may occur are the hydroxyl ion concentration, the sodium and calcium ion concentrations, and the salt concentration of the liquor, and it is of interest to see how these concentrations vary in practice. Values for the composition of lime liquors from various processes are given in Table IV. C is typical of a flat liming process for hides, in which the (OH^-) is higher and the sodium chloride concentration lower than the average, and E represents the other extreme.

TABLE IV.

ANALYSIS OF LIME LIQUORS.

Process		(OH^-) mg. eq. per litre.	Sulphide g.Na_2S per 100 cc.	Sodium mg. eq. per litre	Chloride expressed as g.NaCl per 100 cc.
HIDES.					
Flat Liming ...	C	30–39	0·02–0·11	90–145	0·03–0·85
	E	20–29	0·05–0·13	185–535	1·00–3·00
Suspension Liming ...	C	28–36	0·01–0·06	8–75	0·01–0·06
	E	17–24	0·03–0·10	335–770	1·9 –4·5
Drum Liming ...	1	28–48	0·19–0·39	45–155	0·3 –0·4
	2	36–55	0·21–0·86	25–52	2·0
	3	23–32	0·21–0·88	15–43	3·3
Calf Skins	1	24–68	0·26–0·69	100–210	0·20–0·38
	2	19–36	0·26–0·71	50–125	0·30–0·40
Goat Skins	1	126	2·76	550	—
	2	34	2·76	345	—
Paints	1	850	6·7	1710	—
	2	25	6·8	87	—
	3	25	7·1	—	—

Figures for the suspension liming processes in the same tanneries are also given. Again C is typical of a process with a high (OH^-), and E of a process with a low (OH^-). In none of these processes is the (OH^-) high enough to have any marked effect on cohesion, or to cause any appreciable loss of hide substance.

The sulphide concentration in all four processes is comparatively low and the chief source of sodium ions and salts will be sodium chloride carried in with the hides. Salts, other than sodium salts will be negligible and the sodium ion concentration may be regarded as a measure of the salts concentration of the liquors. In the C processes, the concentration of sodium salts is low and their predominant effect will be to increase the swelling pressure, while in the E processes the salt concentration is sufficiently high to have an appreciable effect on cohesion. In general it is found that in most pit liming processes the sodium ion concentration of the new liquor is sufficient to give the maximum swelling pressure, while in the old liquors the concentration is generally high enough to cause some decrease in cohesion. It has been observed that splitting up of the fibres of the limed pelt, which is an indication of decrease in cohesion, increases as the sodium chloride concentration of the old lime liquor increases. In the pit liming of hides it is probable that the sodium ion concentration of the new liquor should be between 0.2 and 0.6% and that of the old lime should not exceed 2%.

The (OH^-) of the drum liming processes are not excessively high and their salt concentrations are of the same order as the pit liming processes.

The two sets of values for calf skins and goatskins are from experiments on the use of sodium hydrosulphide in liming. The first figures in each case are for the processes using sodium sulphide, and the (OH^-) is considerably higher than that of saturated lime (34 mg. equivalent per litre). The second figures are for similar liquors in which the sodium sulphide was substituted by an equivalent amount of sodium hydrosulphide. The (OH^-) is much lower. The hydroxyl ion concentration of the liquor from the goatskin process is probably

higher than is usually desirable. It is suggested that the (OH^-) should not exceed 100 mg. eq. per litre if loss of hide substance and excessive decrease in cohesion is to be avoided. In any case, it is obvious that control will be difficult in the range where the water uptake increases rapidly with (OH^-).

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Free Amino Groups of Collagen

USING the technique originated by Sanger[1] for the determination of end-groups in proteins by reaction with dinitrofluorbenzene, no free α-amino- or imino-groups have been detected in ox hide collagen[2], even when the equivalent of 2–3 gm. dinitrophenyl-collagen was placed on the silica gel columns.

After heating in water at 70° C., and treating with alkali, or urea, a small number of α-amino groups were found.

Since collagen contains a considerable amount of proline, the dinitrophenyl derivative of which is unstable to acid hydrolysis, special efforts were made to detect this derivative. Recovery of dinitrophenyl-proline added to collagen, and from salmine treated with dinitrofluorbenzene, indicates that proline would have detected it if it had been present to the extent of one end-group per unit of 1,500,000.

Failure to detect an end-group in collagen suggests three possibilities: (1) that the molecular weight is very large—greater than 1,500,000 if proline is an end-group, and of the order of 2,000,000 or more if some other amino-acid forms the terminal group; (2) that the molecule has a cyclic structure; or (3) that the end-group is inaccessible to dinitrofluorbenzene, or is masked in some way by combination with some other group, or with carbohydrate. There is insufficient evidence available at present to indicate which, if any, of these hypotheses is correct; but the following observations may be made. Average molecular weights of up to 250,000 have been reported for gelatin[3]; it is known that a small number of peptide bonds are broken in the conversion of collagen to gelatin, hence the molecular weight of collagen might well be several times this figure. There is only a small amount of carbohydrate in collagen; but it is perhaps of interest to note that the amount of chondroitin sulphate estimated to be present in this collagen from its hexosamine content and the intensity of spots due to glucosamine and chondrosamine on paper chromatograms[4] corresponds approximately to one molecule of chondroitin sulphate per unit of 80,000, that is, in roughly twice the minimum molecular weight (39,000) calculated from the amino-acid composition[2].

In modified collagens, various end-groups were detected in small amounts (see table). With alkali-treated collagen, the small number of end-groups found does not represent the total number of peptide

	α-Amino groups		Reactivity of lysine ε-amino groups with dinitrofluorbenzene (m.mol./100 gm.)
	(m.mol./100 gm.)	Weight containing one residue	
Collagen	No end-groups detected		17
Alkali-treated collagen			
Aspartic acid	0·07	1,400,000	
Glutamic acid	0·17	600,000	
Glycine	0·42	240,000	17
Phenylalanine	0·04	2,500,000	
Collagen dissolved in formic acid			
Aspartic acid	0·43	230,000	
Glutamic acid	0·15	700,000	
Glycine	0·65	150,000	—
Alanine	0·30	330,000	
Urea-treated collagen			
Aspartic acid	0·07	1,400,000	1
Heat-shrunk collagen			
Aspartic acid	0·15	700,000	
Glutamic acid	0·03 (approx.)	3,300,000	15
Gelatin (commercial)			
Aspartic acid	0·28	350,000	
Glutamic acid	0·12	830,000	17
Glycine	0·57	180,000	
Threonine	0·14	710,000	

Lysine content of collagen: 31 m.mol./100 gm.

bonds broken, since about 5 per cent of the collagen was solubilized during the treatment, some as small peptides and some as comparatively large units[5]. These fractions are now being examined. Dispersion in formic acid has often been used as a method of obtaining 'soluble collagen'. The dinitrophenyl derivative of collagen treated with formic acid was soluble and resembled dinitrophenyl-gelatin rather than the other modified collagens.

Aspartic and glutamic acids were frequently found as end-groups of modified collagen, and it would appear that the peptide bonds involving the amino groups of these acids are particularly labile. Partridge[6,7] has also found that these acids are preferentially liberated in the partial hydrolysis of a number of proteins with weak acids.

The amounts of ε-dinitrophenyl-lysine isolated from all the collagens were appreciably less than the amounts corresponding to their lysine contents (see table). Even in gelatin, only about half the ε-amino groups of lysine reacted with dinitrofluorbenzene. Dinitrophenyl-hydroxylysine was also detected; evidence suggests that some of the side-chain amino groups of this amino-acid were also unreactive to dinitrofluorbenzene. Porter[8] also found that the ε-amino groups of a number of soluble

proteins did not react with dinitrofluorbenzene, but that after denaturation all became available. This is not so with collagen, and there is some evidence that heat denaturation actually causes a slight decrease in the number of ε-amino groups reacting. With soluble proteins, denaturation is generally considered to cause unfolding of the polypeptide chains. Collagen is already in the extended form, and heat denaturation, which causes considerable shrinkage, probably involves folding of the chains, which may decrease the availability of amino groups.

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The Reaction of Fluorodinitrobenzene with the α- and ε-Amino Groups of Collagen

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The technique developed by Sanger (1945) for the determination of free amino groups in proteins has been applied to collagen and procollagen with the object of gaining information about the form and size of the molecule. So far this technique has only been applied to a few of the insoluble proteins: wool keratin, myosin and tropomyosin. In keratin a comparatively large number of terminal amino groups were detected suggesting that the molecule consists of a relatively large number of polypeptide chains (Middlebrook, 1951; Blackburn, 1949). With myosin and tropomyosin, however, Bailey (1951) was unable to detect the presence of any terminal amino groups and suggested the possibility of a cyclic molecule.

The evidence available on the structure of collagen from X-ray and electron microscope observations has recently been reviewed by Bear (1952). The fibrils are considered to be formed of regularly coiled polypeptide chains arranged roughly parallel to one another, with regions of similar chemical structure matching transversely. Collagen contains no cystine and there is no evidence for any covalent cross-linkages between the chains, suggesting that, in contrast to keratin, the molecule may consist of a single polypeptide chain. Pauling & Corey (1951),

however, have proposed a structure in which the molecule is considered to be formed of three polypeptide chains, each coiled into a helix and joined to one another by hydrogen bonds.

Porter (1948) has found that, with certain albumins and globulins, not all the ϵ-amino groups react with 1-fluoro-2:4-dinitrobenzene (FDNB) unless the protein is first denatured. With a fibrous protein such as collagen any steric factors affecting the reactivity of such groups are likely to be more pronounced and the non-reactivity may also extend to terminal α-amino groups.

The effect of various treatments on the reactivity of the ϵ-amino groups in collagen towards FDNB and on the liberation of α-amino groups has, therefore, also been considered.

A preliminary account of the work described in this paper was given at the Second International Congress of Biochemistry, Paris, 1952.

MATERIALS

Collagen was prepared from the middle layer of ox hide as described by Bowes & Kenten (1948*a*).

Procollagen was prepared from fresh calf skin as described by Orekhovich, Tustanovskiĭ, Orekhovich & Plotinkova (1948). The calf skin was shaved, cut into small pieces and disintegrated in a large Wiley mill (A. Thomas Co., Philadelphia, U.S.A.). The mill had to be stopped at frequent intervals and the macerated material removed by hand. The macerate (1800 g.) was extracted twice with 10 l. 0·1 M sodium phosphate buffer (pH 8·6) to remove albumins and globulins, and then three times with 10 l. 0·12 M sodium citrate buffer (pH 3·62). All extractions were carried out at 4°. The procollagen was precipitated from the citrate extracts by the addition of NaCl to a final concentration of 5% (w/v). The procollagen was purified by dissolving in slightly acidified water (pH 4·0), and dialysing against tap water.

Modification of collagen

Before treatment the collagen was cut into small pieces, of area about 1 cm.^{2}, or ground in a laboratory-model Wiley mill to pass a 40-mesh/in. sieve.

Heat shrinkage. Pieces of collagen were heated in water at 65–68° for 15 min., cooled and dehydrated with acetone.

Urea treatment. Powdered collagen was immersed in 8 M urea for 24 hr. at laboratory temperature.

Solution in formic acid. Powdered collagen (9·0 g.) was suspended in 500 ml. anhydrous formic acid at 40°. The collagen swelled, and after 18 hr. the greater part had dissolved. The solution was filtered with aid of Celite 545 and Hyflo Super-Cel (Johns-Manville Co., London) and the collagen precipitated by neutralization with 40% (w/v) NaOH. During precipitation the solution was cooled in ice and stirred continuously. The fibrous precipitate was separated and washed with 25% (v/v) aqueous ethanol. The anhydrous formic acid was prepared from the A.R. acid by drying over copper sulphate and distilling under reduced pressure (Garner, Saxton & Parker, 1911).

Alkaline treatment. Pieces of collagen about 0·5 cm.^{2} were immersed in a solution of NaOH and $Ca(OH)_2$ at pH 13·0 for 14 days at 20° (Bowes & Kenten, 1948*b*). Such treatment has been found to dissolve about 5% of the collagen, some as large peptides and some as amino acids and small peptides (Bowes & Kenten, 1948*b*).

Treatment with hyaluronidase. Powdered collagen (5 g.) was treated with half a 1000 unit ampoule of Hyalase (Bengers, Holmes Chapel, Cheshire) dissolved in 150 ml. 0·33 M sodium citrate buffer (pH 4·6) for 16 hr. at 37°. Another 5 g. sample of collagen was treated with heat-inactivated Hyalase under the same conditions. Hyalase is prepared from mammalian testes and may, therefore, be expected to act on chondroitin sulphate as well as hyaluronic acid (Humphrey, 1946; Mathews, Roseman & Dorfman, 1951).

After the enzyme treatment, the collagen was washed by decantation with two 100 ml. portions of the citrate buffer for 8 and 16 hr., followed by two 100 ml. portions of distilled water for similar periods. It was then filtered off, dehydrated with acetone and air-dried.

METHODS

Treatment with fluoro-2:4-dinitrobenzene

Amounts of powdered collagen varying from 1 to 10 g. were treated with FDNB as described by Sanger (1945). For example, 10 g. collagen, suspended in 75 ml. sat. aqueous $NaHCO_3$ and 150 ml. ethanol, were treated with 8 ml. FDNB in a stoppered flask at room temperature. The time of treatment was usually 3 days, but in some cases was extended to 2 or 3 weeks. At the end of the treatment the reaction mixture was decanted off and the collagen thoroughly washed, first with water, then with ethanol and finally with ether. It was then air-dried.

Removal of soluble yellow material, presumably dinitrophenols, etc., by washing with ethanol was very slow, and in later experiments the DNP-protein was Soxhlet-extracted with ethanol for 16 hr. This was found to remove dinitrophenols almost completely.

The dinitrophenyl (DNP) derivatives of gelatin and formic acid-treated collagen were soluble in water and were not readily precipitated by ethanol or acetone. The reaction mixture was neutralized, extracted with ether, and the aqueous layer saturated with $(NH_4)_2SO_4$. The gummy precipitates formed were dialysed first against tap water, and then against distilled water for 3 days, and dried *in vacuo* at room temperature.

Hydrolysis, separation and identification of DNP-amino acids

Two methods of hydrolysis were used: (1) 12 hr. with boiling 6 N-HCl under reflux; (2) 16 hr. in a sealed tube with 10 N-HCl at 105°. Both gave essentially similar results. The latter method was preferred and used for the majority of the experiments since it causes less decomposition of DNP-proline and DNP-hydroxyproline. These residues were considered to be possible end groups in collagen. For the separation of α-DNP-amino acids, 0·5–1 g. of the DNP-protein was usually taken for hydrolysis. Up to 5 g. were taken in attempts to detect a terminal residue in collagen. The hydrolysate was extracted with ether and the α-DNP-amino acids separated on buffered silica gel columns similar to those described by Middlebrook (1949, 1951). The ether extract was first run on a column buffered to pH 6·66

with phosphate buffer (54·6 g. NaH_2PO_4, $2H_2O$ + 53·7 g. Na_2HPO_4, $12H_2O$/l.) and developed with ether saturated with the same buffer solution; this separated glutamic and aspartic acids, serine, threonine and glycine. The faster-moving DNP-amino acids were then separated on a column buffered with the pH 6·66 phosphate buffer and developed with $CHCl_3$ containing 3% (v/v) butanol and saturated with the buffer solution. Additional columns, as described by Sanger (1945) and Blackburn (1949), were used for further separations and confirmation.

For the identification and determination of ε-DNP-lysine and ε-DNP-hydroxylysine smaller amounts (approx. 10–20 mg. of the DNP-protein) were hydrolysed, the hydrolysates were taken to dryness and run on unbuffered silica-gel columns using 66% (v/v) ethyl methyl ketone in ether, or 30% (v/v) *n*-butanol in $CHCl_3$ (Sanger, 1945). In later experiments Hyflo Super-Cel was substituted for silica gel.

The identity of the bands observed was checked by running with authentic DNP derivatives, by paper chromatography of the DNP-amino acids, and of the free amino acids following hydrolysis with saturated $Ba(OH)_2$ for 1 hr. at 105° (Mills, 1950).

The most satisfactory system for the development of paper chromatograms of the DNP-amino acids was found to be *n*-amyl alcohol shaken with an equal volume of 2N ammonia solution, the organic phase being used for development and the aqueous phase for saturation of the cabinet.

Recovery of DNP-amino acids

The recovery of DNP-glycine, DNP-proline and ε-DNP-lysine when added to collagen and hydrolysed in a sealed tube for 16 hr. with 10N-HCl at 105° were 50, 20 and 80%, respectively. The values for DNP-lysine and DNP-glycine were approximately the same as those obtained by Porter & Sanger (1948) under the same conditions, and their values were used in making corrections for the decomposition of the remainder of the DNP-amino acids.

After hydrolysis for 64 hr. only 38% of ε-DNP-lysine added to collagen could be recovered.

Determination of DNP-amino acids

The α-DNP-amino acids were determined colorimetrically in 1% (w/v) $NaHCO_3$ solutions and ε-DNP-lysine in 0·75N-HCl in 25% (v/v) aqueous ethanol.

The amount of DNP-amino acid derived from a given weight of the original collagen was calculated from the weight of the DNP-collagen taken for hydrolysis, corrected for moisture and ash content, for dinitroaniline present, and for DNP groups attached to ε-amino groups. DNP groups attached to terminal amino groups were small in number in all cases, and were neglected. The appropriate corrections were made for decomposition of the DNP-amino acids during hydrolysis.

Determination of lysine and free amino nitrogen

Lysine was determined colorimetrically with ninhydrin following chromatographic separation on an ion-exchange resin (Moore & Stein, 1951).

Free amino nitrogen on the powdered DNP-protein was determined by the Van Slyke procedure using a modified reaction chamber as described by Doherty & Ogg (1943).

RESULTS

Reactivity of ε-amino groups

Both ε-DNP-lysine and ε-DNP-hydroxylysine were identified in hydrolysates of DNP-collagen and DNP-procollagen. The amount of ε-DNP-lysine obtained from DNP-collagen was only equivalent to 17 m-moles/100 g. protein, representing only about half the lysine content. Prolonging the time of reaction with FDNB to 14 or 21 days did not increase the amounts of ε-DNP-lysine found. With procollagen a rather higher proportion of the lysine was recovered as the DNP derivative. ε-DNP-hydroxylysine was not determined quantitatively, but the intensity of the yellow band due to this derivative suggested that the amount present was only equivalent to about half the hydroxylysine content of the collagen.

Following the observation of Porter (1948) that the ε-amino groups of some soluble proteins do not all react with FDNB unless the protein is first denatured, the possibility that some of the ε-amino groups of collagen are also unreactive was investigated. Treatment in 8M urea solution, in alkali, heating in water to 68° or dissolving in formic acid and reprecipitating did not increase the amounts of ε-DNP-lysine found after reaction with FDNB, and there was some suggestion that heat shrinkage actually caused a slight decrease (Table 1). Even with gelatin there was no increase in the amount of ε-DNP-lysine found.

Only small amounts of nitrogen were evolved from all the DNP-proteins by the action of nitrous acid in the usual Van Slyke procedure, and direct determination of lysine in DNP-substituted preparations of collagen and hyaluronidase-treated collagen gave values of only 4 and 3 m-moles/100 g. respectively. These observations suggested that failure to recover all the lysine as the ε-DNP derivative was not primarily due to non-reactivity of a proportion of the lysine to FDNB.

The possibility that the remaining lysine was present as α-DNP-lysine, αε-diDNP-lysine or as resistant DNP-lysine peptides was next considered. No evidence for the presence of these was obtained from the columns, the only unidentified band being a dirtyish yellow one running very slowly on the ether-phosphate buffer column, considered to be a decomposition product of DNP derivatives. Unless a large number of different peptides was formed, relatively large amounts would have to be present to account for 10 m-moles lysine/100 g. protein. Ether-soluble DNP-peptides would, therefore, almost certainly have been detected since, in some instances, the equivalent of 2–3 g. hydrolysed protein was run on the columns. Much smaller amounts were used for the examination of the aqueous extract owing to the large amount of

ε-DNP-lysine present, and small amounts of water-soluble DNP-peptides might not have been detected. Increase in the time of hydrolysis in a sealed tube with 10 N hydrochloric acid at 105° from 16 to 64 hr. caused only a slight increase in the ε-DNP-lysine found (Table 2). Owing to the extensive decomposition of ε-DNP-lysine which occurred during this longer time of hydrolysis, and to the large correction factor which had to be applied, this slight increase was not considered significant.

Terminal amino groups

In addition to yellow bands due to dinitrophenols and dinitroaniline (identified by m.p.) and DNP-amino acids, there was a dirty-yellow material which stayed at the top of the ether-phosphate buffer columns, and a pinkish band running fast on all columns. The first slow band was probably due to some decomposition product of DNP-amino acids, while the fast band which was obtained with hydro-

Table 1. *Free amino groups of collagen and modified collagens*

	α-Amino groups		ε-DNP-lysine found	
	(m-moles/100 g.)	Mol.wt. containing one residue	(m-moles/100 g.)	(% of total lysine residues)
Collagen*	None detected		17	55
Urea-treated				
Aspartic acid	0·07	1 400 000	18	59
Heat-shrunk				
Aspartic acid	0·15	700 000	15	48
Glutamic acid	0·03	3 300 000		
Alkali-treated				
Aspartic acid	0·07	1 400 000	17	55
Glutamic acid	0·17	600 000		
Glycine	0·42	250 000		
Phenylalanine	0·04	2 500 000		
Dissolved in formic acid				
Aspartic acid	0·43	250 000	17	55
Glutamic acid	0·15	700 000		
Glycine	0·65	150 000		
Alanine	0·30	350 000		
Treated with hyaluronidase				
Aspartic acid	0·09	1 100 000	17	55
Alanine	0·16	600 000		
Glycine	0·12	800 000		
Threonine	0·03	3 300 000		
Gelatin (commercial)				
Aspartic acid	0·28	350 000	17	55
Glutamic acid	0·12	830 000		
Glycine	0·57	180 000		
Threonine	0·14	710 000		
Procollagen†				
Aspartic acid	0·06	1 650 000	17	68
Alanine	0·04	2 500 000		

* Lysine content of collagen, 31 m-moles/100 g. † Lysine content of procollagen, 25 m-moles/100 g.

Table 2. *Recovery of ε-DNP-lysine from DNP-proteins*

		m-moles/100 g. DNP-protein			
DNP-protein	Time of hydrolysis* (hr.)	ε-DNP-lysine†	Lysine‡	Free NH_2-N (Van Slyke)	Lysine not accounted for
Collagen	16	17	4	5	10
	16	17	3	—	11
Alkali-treated collagen	16	17	—	—	—
	64	19	—	—	—

* Hydrolysis in a sealed tube with 10 N-HCl at 105°.
† Corrected for decomposition. Recovery of ε-DNP-lysine added to collagen and hydrolysed under the same conditions was 80% and 38% after 16 and 64 hr. respectively.
‡ By method of Moore & Stein (1951).

lysates of collagen as well as DNP-collagen was considered to be due to the protein itself and not to any reaction product of FDNB. The eluate corresponding to this band had a smell of charred sugar.

Collagen. No α-DNP-amino acids were detected in hydrolysates of collagen treated with FDNB, even when amounts equivalent to 2–3 g. protein were placed on the columns. It was estimated that using these amounts most amino acids would be detected if present as a terminal group to the extent of one residue/mol.wt. 4 or 5 million. Recovery tests indicated that DNP-proline, which is the DNP-amino acid least stable to hydrolysis, would have been detected if proline were present as a terminal residue to the extent of one residue/mol.wt. 1 500 000.

Procollagen. Small amounts of DNP-aspartic acid and DNP-alanine were found in hydrolysates of DNP-procollagen. It is possible that these small amounts arise from extraneous matter and do not represent terminal amino groups.

Modified collagens. Small amounts of DNP-amino acids were detected in the hydrolysates of the DNP derivatives of the modified collagens and gelatin (see Table 1). Owing to the number of correction factors involved and the small amounts concerned, the values given in Table 1 can only be considered to be correct to within ± 20 % of the true value.

DNP-Aspartic acid was found in hydrolysates of the DNP derivatives of all the modified collagens and gelatin. With urea-treated collagen this was the only DNP-amino acid found, with heat-shrunk collagen very small amounts of DNP-glutamic acid were also found, and with alkali-treated collagen DNP-glutamic acid, glycine and phenylalanine.

Alanine appeared as an end group when the modification of the collagen involved treatment with acid or slightly acid solutions, e.g. solution in formic acid or extraction with a citrate buffer at pH 4·0 as with procollagen.

The collagen dissolved in formic acid and the commercial gelatin behaved similarly, the DNP derivatives were soluble and the DNP-amino acids found were similar, except that DNP-alanine was found in the formic acid-treated collagen and DNP-threonine in gelatin.

After treatment with hyaluronidase, aspartic acid, alanine, glycine and a trace of threonine were found as terminal residues. No free α-amino groups were found in the collagen treated with the heat-inactivated enzyme under the same conditions, and it seems unlikely that the terminal residues found could have arisen from the hyaluronidase itself. It also seems unlikely that the liberation of α-amino groups from such a variety of amino acids can all be due to removal of polysaccharide actually combined with these groups. It is possible that some hydrolysis may have taken place during the treatment, breakdown of polysaccharide having increased the susceptibility of the peptide bonds to hydrolysis at 37°. In this connexion Jackson (1952) reports that treatment of tendon collagen with hyaluronidase increases its solubility in dilute acetic acid.

DISCUSSION

In studying the reactions of a fibrous protein such as collagen it is always difficult to assess whether the reaction has gone to completion or not. It was hoped that combination of FDNB with the ε-amino groups of lysine could be used as an indication of the course of the reaction. It was found, however, that, although all the ε-amino groups are free to react with nitrous acid (Bowes & Kenten, 1948*a*), ε-DNP-lysine equivalent to only about half these groups was recovered from the DNP-proteins. It was at first thought that this was due to non-reactivity of a proportion of the ε-amino groups towards FDNB as observed by Porter (1948) and others. However, the presence of only small amounts of free lysine in the hydrolysates of DNP-collagen, and the small amounts of nitrogen evolved by nitrous acid in the Van Slyke apparatus indicated that, while it was possible that a few of the lysine groups were unreactive, this could not be the only explanation of the low recovery of ε-DNP-lysine. Out of 31 m-moles lysine/100 g. collagen, approx. 17 were recovered as ε-DNP-lysine (corrected for decomposition during hydrolysis) and 3–4 m-moles were found as unchanged lysine, leaving 10–11 m-moles to be accounted for (Table 2). No evidence was obtained of the presence of α-DNP-lysine, αε-diDNP-lysine or of DNP-lysine peptides in the hydrolysates; increasing the time of hydrolysis did not significantly increase the amount of ε-DNP-lysine found and no yellow bands which might be due to these lysine derivatives were observed on any of the silica-gel columns. There remains the possibility that ε-DNP-lysine is much less stable to acid when combined in the collagen than when present as the free amino acid. In other proteins studied there has been no indication of such decomposition, reasonable agreement being found between the ε-DNP-lysine and the total lysine content of the protein, provided this has been denatured before treatment with FDNB (Sanger, 1952). If extensive decomposition of ε-DNP-lysine does occur, therefore, it would appear to be peculiar to collagen. The possibility that this also applies to other DNP-amino acids should be particularly borne in mind when considering the results of end-group determinations on collagen and modified collagen.

It is interesting to speculate on possible reasons for failure to detect terminal α-amino groups in

collagen. First, the molecule may be very large. Assuming that decomposition of DNP-amino acids during the hydrolytic treatment is no greater when in peptide combination than when free, it was calculated that most terminal amino acid residues would have been detected if present to the extent of one residue/mol.wt. 4 or 5 million. Even if more extensive decomposition occurred it should still have been possible to detect any terminal residue, with the possible exception of proline, if present to the extent of one residue/mol.wt. 1 to 2 million. The molecular weight may, therefore, be of the order of 1 to 2 million at least and probably considerably higher. Average molecular weights of up to 250000 have been reported for gelatin (Pouradier & Venet, 1950), and as some peptide bonds are almost certainly broken during the conversion of collagen into gelatin it is quite possible that the molecular weight of collagen is several times this figure.

The molecule may be cyclic; this does not at first seem to fit in with the conception of a fibrous protein. There is some indication, however, that end groups may be absent in other fibrous proteins, and Bailey (1951), who was unable to detect any end groups in myosin and tropomyosin, has suggested, on the basis of measurements of molecular size (Tsao, Bailey & Adair, 1951), that the tropomyosin molecule is cyclic.

Finally, there is the possibility that the terminal amino groups are inaccessible or masked in some way. The presence of aspartic acid as a terminal residue in collagen which had received such relatively mild treatments as immersion in urea solution or heating to 68° suggests that it may be a terminal residue inaccessible to FDNB in the original collagen. On the other hand, there is evidence that aspartic acid is readily liberated from proteins. Partridge & Davis (1950) and Adair, Partridge & Davis (1951) have shown that aspartic and glutamic acids are preferentially liberated in the partial hydrolysis of a number of proteins with weak acids, and Blackburn (1950*a*, *b*) finds that this also occurs when wool is partially hydrolysed with dilute hydrochloric acid. The free α-amino groups of aspartic acid may, therefore, arise from hydrolysis of peptide bonds during the treatment. In view of the importance now ascribed to mucopolysaccharides in the formation of collagen, the possibility of masking of terminal groups by association with chondroitin sulphate or other polysaccharide is worth consideration. Unfortunately the results of the experiment with testicular hyaluronidase were inconclusive, as it is uncertain whether all the amino groups liberated by this treatment arose from removal of polysaccharide. It may be significant, however, that two of the terminal residues found, aspartic acid and alanine, were also found in procollagen.

There is no evidence of any co-ordinate links in collagen, and it is fairly generally agreed that collagen owes its stability to a large number of interchain hydrogen bonds. Several considerations favour the conception of a molecule consisting of a single long polypeptide chain, and the absence of detectable terminal amino groups is most readily explained on the basis of a very large molecule or the masking of this group, most probably aspartic acid, by association with mucopolysaccharide or some other group. If aspartic acid is not a terminal residue, then the peptide bond involving this amino acid is very labile.

A number of other end groups, in addition to aspartic acid, were found in the modified collagens; these probably arise from hydrolysis of peptide bonds, suggesting that certain peptide bonds involving the amino groups of glutamic acid, glycine, alanine and to a lesser extent threonine and phenylalanine are particularly labile. There is, however, the possibility that some of these DNP-amino acids arise from the uncovering of α-amino groups by the splitting off of DNP-aspartic acid during the treatment with FDNB. This has been found to occur with 'old yellow enzyme' (Weygand & Junk, 1951).

The detection of an appreciable number of free α-amino groups in collagen which has been dissolved in formic acid and reprecipitated calls for some comment. It is often assumed that collagen can be recovered unchanged from formic acid solution, but the present results suggest that the product obtained more nearly resembles gelatin than the original collagen, the number of free α-amino groups actually exceeding those of a commercial gelatin.

SUMMARY

1. The reaction of fluorodinitrobenzene with the amino groups of collagen, modified collagen and procollagen has been studied.

2. Only 55–60% of the lysine could be recovered as the ε-dinitrophenyl (DNP) derivative from collagen, modified collagen or gelatin which had been treated with fluorodinitrobenzene. Only small amounts of free amino nitrogen and unchanged lysine were found in the DNP proteins leaving approx. 30% of the lysine unaccounted for. The possibility of decomposition of ε-DNP-lysine during hydrolysis, and of the presence of acid-resistant ε-DNP-lysine peptides is considered.

3. No terminal residues were detected in collagen, and possible reasons for this are discussed.

4. Small amounts of various end groups were found in procollagen, the modified collagens, and gelatin. Aspartic acid was most frequently found as a terminal residue, followed by glutamic acid,

glycine, alanine and threonine. With the possible exception of aspartic acid these are considered to arise from hydrolysis of peptide bonds during the treatments. Aspartic acid is either a terminal residue which is inaccessible to fluorodinitrobenzene or the peptide bond involving its α-amino group is especially labile.

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SOME DIFFERENCES IN THE COMPOSITION OF COLLAGEN AND EXTRACTED COLLAGENS AND THEIR RELATION TO FIBRE FORMATION AND DISPERSION

By

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SOME DIFFERENCES IN THE COMPOSITION OF COLLAGEN AND EXTRACTED COLLAGENS AND THEIR RELATION TO FIBRE FORMATION AND DISPERSION

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A few years ago Orekhovitch and co-workers (1948a, 1952) extracted a soluble protein from the skin of various animals using citrate buffers of pH 3 to 4. The composition of this protein resembled collagen, and since there was more present in young animals than old, they suggested it was a precursor of collagen, and named it *procollagen.*

A soluble protein has been extracted from calf skin under the conditions described by Orekhovitch, Toustanovski, Orekhovitch and Plotnikova (1948a), and its amino acid composition has been determined.

Certain differences in the composition of this citrate extracted protein and that of ox hide collagen suggested that, if it is indeed a precursor of collagen, then its conversion to collagen involves the addition of a protein fraction relatively rich in tyrosine and certain other amino acids, and relatively low in hydroxyproline, alanine and serine compared with collagen.

Some experiments on the treatment of ox hide collagen in alkaline solutions suggested that the material going into solution also contained a high proportion of tyrosine and very little hydroxyproline. It was, therefore, thought that it would be interesting to see whether there was any suggestion that dispersion in general involved the splitting off of some specific fraction similar in composition to that which appears to be added to *procollagen* to obtain collagen. With this object in view collagen has been given various treatments favouring dispersion and the amino acid composition of the treated collagen, and of the protein going into solution during the treatment has been studied.

Preliminary observations on the hydroxyproline, tyrosine and hexosamine contents of various modified collagens are reported.

EXPERIMENTAL

Preparations

Citrate extracted protein—A soluble protein was extracted from fresh calf skin as described by OREKHOVITCH, TOUSTANOVSKI, OREKHOVITCH and PLOTNIKOVA (1948a). The skin was shaved, cut into small pieces and disintegrated in a Wiley mill. The macerate (1800 g) was extracted twice with 10 litres of 0·1 M sodium dihydrogen phosphate solution, pH 8·6, and then three times with 10 litres of 0·12 M sodium citrate solution, pH 3·62.

The protein was precipitated from the citrate extracts by the addition of sodium chloride to a final concentration of 5 per cent (w/v). The precipitated protein was purified by dissolving in slightly acidified water, pH 4·0, and dialysing against tap water. It was acetone dehydrated and air dried. The ash content of the final product was 0·5 per cent and the moisture content 16·5 per cent.

Collagen—Collagen was prepared from ox hide as previously described (BOWES and KENTEN, 1948).

Treatments

Alkali—Ox hide collagen was treated in a solution of sodium and calcium hydroxides at pH 13·0 for 14 days (see BOWES and KENTEN, 1948). About 5 to 10 per cent of the protein dissolved during the treatment; this was separated into two fractions by neutralization and precipitation with acetone.

Hyaluronidase—Powdered collagen (5 g) was treated with half an ampoule of Bengers Hyalase dissolved in 150 ml 0·33 M sodium citrate buffer pH 4·6 for 16 h at 37°C. The collagen was washed by decantation with two 100 ml aliquot portions of citrate buffer for 8 and 16 h, followed by two 100 ml aliquot portions of distilled water. It was filtered off, dehydrated with acetone and air dried.

Acetic acid—About 4 g fresh rat tail tendon was treated for 3 days in 500 ml 0·05 per cent acetic acid solution adjusted to pH 2·4 with hydrochloric acid. The solution was centrifuged and filtered. The residue was washed and dehydrated with ethanol, and the protein in solution was precipitated by the addition of sodium chloride to a final concentration of 5 per cent (w/v). The total nitrogen, hydroxyproline and tyrosine contents of the various fractions were determined.

Methods

The amino acid composition of the citrate extracted protein was determined by the method of MOORE and STEIN (1951b) involving

separation on an ion exchange resin followed by colorimetric determination with ninhydrin.

Hydroxyproline was determined by the method of NEUMAN and LOGAN (1950a). The accuracy and reproducibility of the method was not as great as could have been desired, but all determinations were carried out under the same conditions and it was considered that the results were comparable. Replicate determinations did not vary from the average value by more than ±5 per cent. Tyrosine was determined on an acid hydrolysate by LUGG's (1937, 1938) modification of the Millon-Weiss method (WEISS, 1919).

Hexosamine was determined by the ELSON and MORGAN (1933) method as modified by PALMER, SMYTHE and MEYER (1937). Results were reproducible to ±5 per cent, but tended to be high; recovery of hexosamine added to collagen varied between 105 and 112 per cent.

Total nitrogen and amino nitrogen were determined as previously described (BOWES and KENTEN, 1948).

Results

The amino acid composition of the soluble protein extracted by citrate buffer as determined by the method of Moore and Stein is given in *Table I.* Data for ox hide collagen taken from the paper by BOWES and KENTEN (1948) are given for comparison.

Although the composition of the extracted protein is in general similar to that of collagen, there are appreciable differences in the amounts of certain of the amino acids; these are underlined in *Table I.*

The total nitrogen is rather lower than that of collagen, partially accounted for by the lower amide, histidine and lysine contents. Other amino acids which are low compared with collagen are tyrosine and proline, while the amounts of hydroxyproline, alanine and serine are relatively high.

It is uncertain whether the glutamic acid content of the extracted protein is really higher than that of collagen. The value given in *Table I* for collagen was determined some years ago by the method described by CONSDEN, GORDON and MARTIN (1948). It was thought at the time to be low and some preliminary determinations by the Moore and Stein method seem to confirm this.

Although the hexosamine : nitrogen ratio of the citrate extract was relatively high, only a small proportion of this was precipitated with the protein, and the hexosamine content of the extracted collagen was less than that of the collagen.

Table I. Composition of Collagen and Citrate Extracted Collagen

Material	*Collagen* g/100 g	*Citrate extracted collagen* g/100 g	*Amino acid nitrogen as* per cent *total protein nitrogen*	
			Collagen	*Citrate extracted collagen*
Total nitrogen	18·60	17·70	—	—
Amino nitrogen	0·46	0·49	2·5	2·7
Glycine	26·2	29·1	26·3	30·7
Alanine	9·5	11·9	8·0	10·5
Leucine	5·6	3·4	3·2	2·1
Isoleucine		1·6		0·9
Valine	3·4	2·8	2·2	1·9
Serine	3·4	4·5	2·5	3·4
Threonine	2·4	2·3	1·5	1·5
Methionine	0·8	0·9	0·4	0·5
Cystine	0·0	—	0·0	—
Proline	15·1	12·7	9·9	8·7
Hydroxyproline	12·2	14·0	7·0	8·4
Phenylalanine	2·5	2·2	1·1	1·1
Tyrosine	1·4	0·6	0·6	0·25
Tryptophan	0·0	—	0·0	—
Arginine	8·8	8·8	15·3	16·0
Histidine	0·8	0·4	1·2	0·6
Hydroxylysine	1·3	1·0	1·2	1·0
Lysine	4·5	3·8	4·7	4·1
Aspartic acid	6·3	6·6	3·6	3·9
Glutamic acid	11·3	12·1	5·8	6·5
Amide nitrogen	0·66	0·52	3·5	2·9
Hexosamine	0·33	0·19	0·14	0·08

OREKHOVITCH (1952) has already drawn attention to the low tyrosine content of his *procollagen* and has suggested that there is a series of proteins gradually increasing in tyrosine content and decreasing in ease of extractability until finally collagen fibres are obtained. The observations reported above indicate that the conversion of this soluble protein to insoluble collagen fibres would require the addition of a protein fraction relatively rich in tyrosine, histidine, lysine, proline, and amide nitrogen, and relatively low in alanine, serine and hydroxyproline. Since the hexosamine content of the soluble protein was also lower than that of collagen, this fraction might be a mucopolysaccharide.

COMPOSITION OF TREATED COLLAGENS

Alkaline treatment

Collagen does not dissolve to any great extent in alkali, but the extensive swelling which occurs, and the increased readiness with which it can then be converted into gelatin are evidence that such treatment favours dispersion of the collagen. In the present experiment between 5 and 10 per cent of the collagen dissolved during the treatment, about one quarter of which was precipitated by the addition of acetone. Amino nitrogen determinations indicated that the precipitated fraction consisted of large polypeptides, while the more soluble fraction consisted mainly of small peptides and free amino acids.

The hydroxyproline, tyrosine and hexosamine contents of some of the fractions are given in *Table II.*

Table II. Composition of Treated Collagens

Material	*Hexosamine* g/100 g	*Hydroxyproline* g/100 g	*Tyrosine* g/100 g
Collagen	0·33	12·2	1·0
Citrated extracted collagen	0·19	14·0	0·65
Alkali treated collagen:			
(*1*) *Insoluble*	0·17	13·5	0·3
		Paper chromatograms	
(*2*) *Precipitated by acetone*	—	*Medium*	*Very faint*
(*3*) *Not precipitated by acetone*	—	*Absent*	*Relatively strong*
Gelatin	—	14·0	0 *to* 1·1
Hyaluronidase treated collagen	—	12·5	0·53

The alkali treated collagen contained less tyrosine and hexosamine and a higher proportion of hydroxyproline than the original collagen, the values approaching those for the citrate extracted protein. A commercial gelatin prepared from alkali treated collagen was also found to have a high hydroxyproline content and the average of values quoted in the literature for tyrosine in gelatin (0·3 per cent) is appreciably lower than the corresponding average value for collagen (1·0 per cent).

The material precipitated by acetone gave a paper chromatogram similar to that of collagen except that the hydroxyproline spot was relatively weak and the tyrosine spot slightly more marked than is

usual with collagen. Paper chromatograms of the more soluble fraction showed no hydroxyproline spot and tyrosine was relatively strong compared with collagen.

Thus, the results indicate that during the alkaline treatment of collagen a fraction is split off which is relatively rich in tyrosine and hexosamine and which contains little hydroxyproline, leaving an insoluble material similar in composition to the citrate extracted collagen with respect to these constituents. The amino acid composition of these fractions is being examined in more detail.

Hyaluronidase treatment

Treatment with hyaluronidase has been reported to increase the swelling of collagen and its solubility in dilute acetic acid, due, it is suggested, to removal of chondroitin sulphate (JACKSON, 1952).

The hydroxyproline content of the treated protein was slightly higher than that of collagen and the tyrosine content was appreciably lower.

Solution in acetic acid

Collagen dissolves fairly readily in anhydrous formic and acetic acids and to a limited extent in the dilute acids depending on the state of organization of the collagen, tendon dissolving more readily than skin. Extensive swelling appears to be a necessary preliminary to solution in the dilute acids, the protein apparently dissolving in its own adsorbed water.

Rat tail tendon was used in this experiment in preference to ox hide collagen because of its greater solubility in acetic acid. About nine tenths of the tendon dissolved during treatment in the 0·05 per cent acetic acid solution. Nearly all of this was precipitated by the addition of sodium chloride giving a material similar in appearance and behaviour to the citrate extracted protein.

Table III. Solution of Tendon in Acetic Acid

Material	*Hydroxyproline*	*Tyrosine*
	Nitrogen as per cent *total nitrogen*	
Control. Wallaby tendon . . .	6·8	0·40
(*1*) *Insoluble fraction* . . .	7·1	*Faint on paper chromatogram*
(*2*) *Soluble in acetic acid* . .	7·1	—
(*3*) *Fraction precipitated by sodium chloride*	8·1	0·18

The hydroxyproline and tyrosine contents of the different fractions expressed as amino acid nitrogen as a percentage of total nitrogen are given in *Table III*. Values for untreated wallaby tail tendon are given for comparison.

The hydroxyproline content of the insoluble fraction and of the acetic acid solution were the same, suggesting that the tendon as a whole goes into solution, the extent probably depending on the conditions. The hydroxyproline content of the precipitated protein, however, was relatively high, approaching that of the citrate extracted protein. The tyrosine content was low compared with the value for the wallaby tendon.

DISCUSSION

The differences observed in the amino acid composition of skin collagen and the protein extracted from the skin by citrate buffers suggests that any conversion of this soluble protein to collagen fibres must involve the addition of a protein fraction relatively rich in tyrosine, histidine, lysine, proline and amide nitrogen, and relatively low in hydroxyproline, alanine and serine. Since the hexosamine content of the extracted protein was also low this fraction might be a mucopolysaccharide. Changes in the tyrosine, hydroxyproline and hexosamine contents also appear to be involved in the solution of collagen, conditions favouring dispersion resulting in the splitting off of a fraction low in hydroxyproline and relatively rich in tyrosine and hexosamine, and leaving a protein approaching the composition of the citrate extracted protein with respect to these constituents.

On the basis of these observations it is tentatively suggested that fibre formation may involve the association of soluble protein, resembling the citrate extracted material, with a mucopolysaccharide fraction; and that dispersion involves the reverse process, the dissociation of this mucopolysaccharide fraction. The mucopolysaccharide may form part of the molecule or may act more in the nature of a cementing substance. No N-terminal residues have been found in collagen (Bowes and Moss, 1951), but aspartic acid and alanine have been found as terminal residues in the citrate extracted protein and in collagen treated with hyaluronidase (Bowes and Moss, 1953). Aspartic acid has also frequently been found in collagen which has received relatively mild treatments, and it is possible that the amino group of this amino acid is involved in the association of mucopolysaccharide.

Table IV. Hydroxyproline and Tyrosine Contents of Fibrinoids in Rheumatic Fever and Rheumatoid Arthritis

Material	*Nitrogen as* per cent *total nitrogen*		
	Hydroxyproline	*Tyrosine*	*Reducing sugars*
In rheumatic fever (CONSDEN)			
Normal tissue—extract . .	8·2	0·7	1·3
Fibrinoid—extract . . .	7·6	1·6	2·9
Normal tissue—residue . .	1·1	1·6	—
Fibrinoid—residue . . .	0·7	3·4	2·7
In rheumatoid arthritis (ZIFF)			
Material extracted from fibrinoids by alkali	*None*	*High*	7 per cent *polysaccharide*

It is interesting to find that changes in the hydroxyproline, tyrosine and polysaccharide contents of collagenous tissue have also been observed in vitamin C deficiency and in rheumatic complaints (*Table IV*). ROBERTSON and SCHWARTZ (1953) found that in the absence of vitamin C the collagen-like material formed around Irish moss injected into guinea pigs was apparently more soluble in dilute alkali and contained less hydroxyproline than that formed in normal animals. Other workers have reported increased formation of polysaccharide (BUNTING and WHITE, 1950; BRADFIELD and KODICEK, 1951).

CONSDEN *et al.* (1952) also report differences in the tyrosine, hydroxyproline and reducing sugar contents of fibrinoids formed in rheumatic fever compared with normal subcutaneous tissue. The solution obtained on autoclaving, assumed to be gelatin from the original collagen, was lower in hydroxyproline and higher in tyrosine and reducing sugar than the corresponding solution obtained from normal tissue. The residue obtained from the fibrinoids was also lower in hydroxyproline and higher in tyrosine than the similar residue obtained from normal tissue.

ZIFF (1952) has also found that the amount of material extracted by dilute alkali from the fibrinoids found in rheumatoid arthritis was greater than with normal tissue, and contained 7 per cent polysaccharide, no hydroxyproline and a high proportion of tyrosine.

Thus, in the formation of abnormal collagenous tissue there appears to be an increase in material relatively soluble in alkali and which is low in hydroxyproline and rich in tyrosine and

polysaccharide, *i.e.* similar in these respects to the fraction which goes into solution when collagen is treated under conditions favouring dispersion.

SUMMARY

Certain differences in the amino acid composition of the protein extracted from calf skin by citrate buffers (the *procollagen* of Orekhovitch) and of ox hide collagen suggest that any conversion of the citrate extracted protein to collagen fibres involves the addition of a fraction relatively rich in tyrosine and certain other amino acids, and low in hydroxyproline, alanine and serine.

Consideration of the factors influencing the dispersion of collagen, and the examination of the soluble and insoluble fractions obtained on treating collagen with alkali or dilute acetic acid indicate that dispersion involves the reverse process, the removal of a fraction rich in tyrosine and hexosamine and low in hydroxyproline.

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The Composition of Collagen and Acid-Soluble Collagen of Bovine Skin

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The partial dissolution of collagen in dilute solutions of weak acids, such as formic and acetic, has been known for some years, and has been extensively studied by Nageotte (1927*a*, *b*, 1928, 1930, 1933), Nageotte & Guyon (1933), Leplat (1933), and Fauré-Fremiet (1933). Interest in this soluble protein has been revived in recent years by the work of Orekhovich and his colleagues in the U.S.S.R., who report the extraction of a soluble collagenous-type protein from the skin of various animals using dilute citrate buffers, and which they suggest is a soluble precursor of collagen (Plotnikova, 1947; Tustanovskii, 1947; Orekhovich, Tustanovskii, Orekhovich & Plotnikova, 1948; Chernikov, 1949; Orekhovich, 1950, 1952). The information regarding these various soluble collagens has recently been reviewed by Harkness, Marko, Muir & Neuberger (1954). These authors also report the presence of a small amount of a protein of collagen type which is extracted from skin by dilute phosphate, pH 9·0 (alkali-soluble collagen). On the basis of experiments on the feeding of labelled glycine to rabbits they conclude that this is a true precursor of collagen, whereas the metabolic role of the acid-soluble collagen described by Orekhovich is less certain, and it is not necessarily an intermediate in the formation of all the insoluble collagen of the skin.

Harkness *et al.* (1954) determined the hydroxyproline and tyrosine content of the alkali-soluble and acid-soluble collagen, and also of the gelatin obtained from the remaining insoluble collagen. Both soluble collagens contained less tyrosine and more hydroxyproline than the insoluble collagen, and the acid-soluble had a higher hydroxyproline and tyrosine content than the alkali-soluble collagen. Similar differences have been observed by Bowes, Elliott & Moss (1953) between the hydroxyproline and tyrosine content of the acid-soluble collagen of calf skin and the adult collagen of ox hide; and between the acetic acid-soluble and insoluble fractions of tendon collagen.

A complete analysis of the acid-soluble collagen of calf skin has now been carried out in order that a more detailed comparison may be made with that of adult collagen. A preliminary account of some of these results has already been given (Bowes *et al.* 1953). The chromatographic method of Moore & Stein (1951) was used for the determination of the amino acids, and it was thought

desirable to repeat the analysis of ox-hide collagen using this method, so that direct comparison of the two proteins could be made.

In view of the apparent influence of polysaccharide on the dispersion of collagen in dilute acid solutions (Jackson, 1953) some further investigations on the sugars and amino sugars present in collagen and acid-soluble collagen have also been made.

EXPERIMENTAL

Preparation of proteins

The ox-hide collagen was from the same batch as that used in previous investigations (Bowes & Kenten, 1948*a*, *b*). It was originally prepared from the middle layer of a hide taken from a 2-year-old bullock immediately after flaying.

The acid-soluble collagen was prepared from the skin of a 6- to 8-month-old bull calf by the method described by Orekhovich *et al.* Immediately after flaying the skin was shaved, cut into pieces about 1 cm.^{3} and disintegrated in a Wiley mill (A. Thomas and Co. Philadelphia). It was necessary to stop the mill at intervals and remove the macerate by hand since it would not go through even the coarsest sieve. The macerated material (1800 g.) was placed in a cotton bag and extracted as indicated in Table 1, first with 0·1 M-Na_2HPO_4, pH 8·6, and then with 0·12 M sodium citrate buffer, pH 3·62.

After the last phosphate extraction the macerate was suspended for a short time in two 3-l. portions of the citrate buffer in order to remove the phosphate buffer before going on to the citrate extractions proper. Each extraction was for a period of 24 hr., during which time the contents of the bag were agitated intermittently. All extractions were carried out between 2 and 4°. The macerated skin swelled considerably during the extractions; as much liquid as possible was squeezed out after each extraction, but relatively large amounts remained behind. After the last extraction special efforts were made to squeeze out liquid, and the relatively high protein content of this extract suggests that some liquid remaining from the earlier extractions was removed from the interstices of the protein. A small amount of the macerate (5 g.) was further extracted with successive 200-ml. portions of the citrate buffer. Samples were taken at various stages, dehydrated with acetone, and total nitrogen and hexosamine determinations were carried out.

The protein in the citrate extracts was precipitated by the addition of sufficient 30 % (w/v) sodium chloride solution to bring the final concentration to 5 % (w/v). The next morning the lower clear layer of liquid was withdrawn, and the top layer, containing the protein in a gelatinous form, was centrifuged. The precipitate was washed with a small amount of water and dehydrated with acetone, it having first been ascertained that this did not apparently alter the solubility of the extracted protein. The precipitated protein was further purified by redissolving in citrate buffer and dialysing against tap water.

The citrate-soluble collagen was converted into gelatin by heating a 2 % (w/v) suspension in slightly acidified water to 40° for 5 min. The protein went into solution during the heating and on cooling the solution set to a gel.

Chemical analyses

Total nitrogen. This was determined according to the method of Chibnall, Rees & Williams (1943).

Amide-nitrogen. The method described by Bailey (1937) and Lugg (1938*c*) was used.

Amino-nitrogen. The free amino-nitrogen was determined by the Van Slyke manometric procedure using a modified reaction chamber as described by Doherty & Ogg (1943).

Amino acids. With the exception of hydroxyproline, amino acids were determined colorimetrically with ninhydrin following separation on an ion-exchange resin (Moore & Stein, 1951). The columns used were of slightly greater diameter (1·0 cm.) than those used by Moore & Stein; this resulted in the complete separation of tyrosine and phenylalanine on the 100 cm. column, and the partial separation of hydroxylysine and histidine on the 15 cm. column used for the separation of the basic amino acids. The length of this column was increased to 20 cm. to improve this separation, and that of a third constituent eluted in the same range.

The individual peaks were identified by comparison with the behaviour of known amino acid mixtures run on similar columns and, in some instances, by paper chromatography.

In the colorimetric determination 2 ml. of ninhydrin were used. In order to keep the blank readings low it was necessary to redistil the methoxyethanol, and to recrystallize the ninhydrin. The citrate buffer used for making up the ninhydrin was shaken up with Dowex-50 resin (sodium form) to remove ammonia, and stored over this resin. Ethanol was used in place of propanol in the diluent. Several runs were made on each protein using different hydrolyses, at least two and in many cases up to four determinations being made of each amino acid.

For the majority of the amino acids the estimated error was ±3 % or less. In particular, for the amino acids for which differences between collagen and citrate-soluble collagen are reported, the estimated errors were: tyrosine ±4 %; leucine ±2 %; isoleucine ±3 %; aspartic acid ±4 %. Rather greater errors were obtained with lysine (±5 %), valine, serine and threonine (±6 %) and methionine (±8 %). These greater errors are probably due to variable losses during hydrolysis and, in the case of valine, to the small amount present and the rather flat peak which it gives. Values for histidine and hydroxylysine were also rather variable owing to the considerable overlap of the peaks in this region. Values were chosen from the runs which gave the most satisfactory separation.

Two different methods of hydrolysis were used: conc. HCl in a sealed tube at 105° for 16 hr. and 6 N-HCl under reflux for 24 hr. There was no evidence of any variations due to the method of hydrolysis.

Hydroxyproline. The method described by Neuman & Logan (1950) was used. Experiments carried out on the method of hydrolysis showed no evidence that hydrolysis under pressure (25 lb./in.^{2}) with 6 N-HCl for 6 hr., under reflux with 6 N-HCl for 24 hr. or 48 hr., or in a sealed tube with conc. HCl at 105° for 16, 24 or 48 hr. gave essentially different results. In all the experiments recorded, about 200 mg. protein were hydrolysed with 10 ml. conc. HCl in a sealed tube at 105° for 16 hr. and the hydrolysate made up to 1 l. At this dilution no neutralization of the 1 or 2 ml. sample taken for the determination was necessary. In the colorimetric determination the volumes of the reagents used

by Neuman & Logan were doubled, and after development of the colour the final volume was made up to 25 ml. with distilled water. The concentration of $CuSO_4$ was increased to 0·05 M as recommended by Baker, Lampitt & Brown (1953). Readings were made on a Hilger Spekker absorptiometer using 2-cm. cells and an Ilford filter, no. 605 (max. transmission, 530–575 mμ.). Determinations were carried out in triplicate on each of two separate hydrolyses. In each determination four samples of the unknown were treated together with four standards in duplicate. Standard deviation for determinations on collagen 0·14 and for citrate-soluble collagen 0·18.

In making earlier determinations it was observed that the rate at which the H_2O_2 was added was important. If the peroxide was added slowly with shaking the optical density of the hydroxyproline standards was greater than when it was added rapidly, but that of the unknowns containing the protein hydrolysate was not always similarly affected. Consequently low, and variable, values for the unknowns were often obtained. In all the determinations recorded the peroxide was added rapidly and the flasks shaken three or four times over a period of 5 min.

It was also observed that with some batches of propanol the colour was found to fade relatively rapidly, and these were discarded.

Hexosamine. The method of Elson & Morgan (1933) as modified by Blix (1948) was used. The reproducibility of the determination was ±5% and the recovery of glucosamine added to collagen was high (105–110%). In subsequent experiments it was found that there was considerable interference from the other constituents of the hydrolysate (see Appendix).

The values recorded in Table 2 are uncorrected for this interference, but although too high are probably comparable with one another.

Total and sulphate sulphur. Total S was determined by the method of Barritt (1934) modified to deal with small amounts of sulphur. Sulphate S was determined by a modification of the method of Lugg (1938*a*). Owing to the very small amount of sulphate S present in collagen, it was necessary to adapt the method to a microscale and to make corrections for sulphate present in the reagents used. Recovery of sulphate added to collagen was 93%, and the appropriate corrections were made to the values obtained for collagen alone. In view of the very small amounts present, and the larger amount of protein required, these determinations were not repeated on the citrate-soluble protein.

Free amino groups. The technique described by Sanger (1945), using 1-fluoro-2:4-dinitrobenzene, was used for the identification and determination of the free amino groups. Details of the methods of treatment, separation and identification have already been described (Bowes & Moss, 1953).

RESULTS

Extraction of skin

The nitrogen extracted with phosphate buffer represents about 5·5% of the total nitrogen of the macerated skin (Table 1). Protein was precipitated on the addition of ammonium sulphate to half and full saturation, and was assumed to consist of albumins and globulins, respectively. This fraction was not examined further.

Table 1. *Extraction of calf skin* (1800 *g.*)

For details see text.

Extracting buffer	Volume added to macerate (litres)	Volume recovered from macerate (litres)	Nitrogen content of extracts (mg./ml.) Before pptn.	Nitrogen content of extracts (mg./ml.) After pptn.	Nitrogen as % total nitrogen of macerate: Total extracted	Nitrogen as % total nitrogen of macerate: Extracted and pptd.
0·1 M-Na_2HPO_4 (pH 8·6)						
1	10·0	8·6	0·19	—	1·95	—
2	10·0	8·3	0·36	—	3·55	—
				Total	5·50	—
0·12 M Citrate (pH 3·62)						
1	10·0	9·2	0·19	0·05	2·08	1·47
2	10·0	11·3	0·07	0·03	0·94	0·46
3	3·0	3·5	0·44	0·08	1·91	1·52
				Total	4·93	3·45
Further extractions on 5 g. macerate	(ml.)	(ml.)				
4	200	—	0·08	—	—	—
5	200	—	0·05	—	—	—
6	200	—	0·02	—	—	—

In six further extracts the nitrogen varied from 0·022 to 0·015 mg./ml.

A similar proportion of the total nitrogen (4·9%) was extracted in the first three main citrate extractions. Further small amounts were extracted in the subsequent treatments of a 5 g. portion, the amounts finally becoming approximately constant after the sixth extraction at about 0·02 mg. nitrogen per ml.

On the addition of sodium chloride to the three citrate extracts between 50 and 80% of the protein

was precipitated. The protein remaining in solution probably consisted partly of albumins and globulins not completely removed by the phosphate buffer, since the addition of ammonium sulphate precipitated a further 10 % of the total nitrogen of the extract. Some of the citrate-soluble collagen also probably remained in solution since the precipitation is unlikely to be complete. There is also the possibility of an additional protein fraction, for it has since been found that 10–30 % of the nitrogen in citrate buffer and acetic acid solutions of tendon collagen is not precipitated by sodium chloride, even after continued extraction makes the presence of albumins and globulins from the tissue fluids extremely unlikely. This nitrogen was in the main non-dialysable, and a preliminary analysis indicated that the hydroxyproline content was negligible, the glycine, alanine and proline contents were relatively low, and the tyrosine, leucine, aspartic acid and amide contents high. It would appear, therefore, to represent a non-collagenous fraction.

The hexosamine content of the macerate was unaffected by the phosphate extractions, but was appreciably decreased by the treatments with citrate buffer (Table 2). The citrate extract contained a relatively high proportion of hexosamine in relation to nitrogen, but only a small amount of this was carried down with the precipitated protein, the hexosamine/nitrogen ratio of the solution being increased to 0·11. These hexosamine determinations were only corrected for the colour of the hydrolysate in the absence of added Ehrlich's reagent, and not for interference of other constituents of the hydrolysate.

Table 2. *Nitrogen and hexosamine content of extracts and skin*

Material	Hexosamine*	Nitrogen	Hexosamine / Nitrogen
Calf skin:	(g./100 g. moisture- and ash-free protein)		
After maceration	0·41	17·66	0·023
After phosphate extraction	0·42	—	—
After citrate extraction	0·34	17·85	0·019
Precipitate from citrate extract:			
Citrate-soluble collagen	0·19	17·70	0·011
First citrate extract:	(mg./100 ml. extract)		
Before precipitation	0·78	19·2	0·046
After precipitation	0·54	4·9	0·110

* Uncorrected values see p. 145.

Properties of citrate-extracted protein

The precipitate formed on the addition of sodium chloride to the citrate extract of calf skin was fibrous in appearance and, when observed under the microscope (phase contrast), the formation of definite fibre-like particles could be seen. Some of the staining reactions of this fibrous network are listed in Table 3. The precipitate was fixed in Zenkers' fixative ($K_2Cr_2O_7 + HgCl_2 +$ acetic acid). It would appear that the staining properties lie between those of collagen and reticular tissue.

Table 3. *Staining reactions of citrate-soluble collagen*

Stain	Reaction
Iodine	Red brown—more deeply staining than collagen
Periodic acid-Schiff	Faintly positive like collagen
Azan and Mallory's triple stain	Dense blue like collagen and reticulin
Haem alum and basic stains	Not stained, resembling collagen and reticulin
Silver	Purple to black—like reticulin rather than collagen
Toluidine blue	Not metachromatic

The protein was soluble in dilute buffers or slightly acidified water to the extent of about 0·5 g. per 100 ml. Solubility was slightly increased in the presence of small amounts of salts and decreased with rise in pH.

On heating in water above 40° for a few minutes and cooling, the solution set to a gel, owing presumably to conversion into gelatin. The rigidity of such a gel containing the equivalent of 2 g. protein in 100 ml. 0·05 M acetate buffer at pH 5·0 was determined by Mr P. R. Saunders of the British Gelatine and Glue Research Association. The determination was made at 10° after maturing for 17 hr. at the same temperature and a value of 12 000 dynes/cm.^{2} was obtained. Gelatins of similar rigidity have been prepared in the B.G.G.R.A. Laboratories from skin and tendon, but high-grade commercial gelatins under the same conditions have lower values of the order of 9000 dynes/cm.^{2}.

Composition of collagen and procollagen

The amino acid composition of adult ox-hide collagen, and of the citrate-soluble collagen of calf skin, both determined by the technique of Moore & Stein (1951), are given in Table 4. The composition of the same ox-hide collagen as reported in 1948 (Bowes & Kenten, 1948*a*) is also given for comparison.

agrees better with the Van Slyke value for free amino nitrogen than did the previous figure, and the new value for arginine is only slightly lower than that obtained by flavianate precipitation (14·40 %) on the same material (Bowes & Kenten, 1948*b*). It was suggested at the time (Bowes & Kenten, 1948*a*) that the values for the dicarboxylic acids determined by the method of Consden, Gordon & Martin (1948) might be low, and the

Table 4. *Amino acid content of ox-hide collagen and of citrate-soluble collagen of calf skin*

	Citrate-soluble protein				Ox-hide collagen				Ox-hide collagen as reported 1948*	
	N as % protein N	g./100 g.	g. residues/ 100 g.	m-moles/g.	N as % protein N	g./100 g.	g. residues/ 100 g.	m-moles/g.	N as % protein N	g./100 g.
Total N	—	17·70	—	—	—	18·60	—	—	—	18·60
Amino N	2·65	0·49	—	0·35	2·50	0·46	—	0·33	2·50	0·46
Glycine	27·48 (4)	26·07	19·81	3·47	26·66 (2)	26·57	20·20	3·540	26·3	26·2
Alanine	8·84 (4)	9·95	7·94	1·17	8·72 (2)	10·32	8·23	1·158	8·0	9·5
Leucine	1·93 (3)	3·20	2·76	0·244	2·14 (2)	3·73	3·22	0·284	3·2	5·6
Isoleucine	0·84 (3)	1·39	1·20	0·106	1·08 (2)	1·88	1·62	0·143		
Valine	1·53 (2)	2·26	1·91	0·193	1·58 (2)	2·46	2·08	0·210	2·2	3·4
Phenylalanine	0·95 (3)	1·98	1·78	0·120	1·07 (2)	2·35	1·95	0·142	1·1†	2·5
Tyrosine	0·22 (2)	0·50	0·45	0·028	0·41 (2)	0·99	0·89	0·054	0·6	1·4
Tryptophan	—	—	—	—	—	—	—	—	—	—
Serine	3·19 (4)	4·23	3·51	0·403	3·06 (4)	4·27	3·54	0·406	2·5	3·4
Threonine	1·47 (3)	2·21	1·87	0·185	1·43 (4)	2·26	1·92	0·190	1·5	2·4
Cystine	—	—	—	—	—	—	—	—	—	—
Methionine	0·41 (3)	0·78	0·68	0·052	0·49 (2)	0·97	0·85	0·065	0·4	0·8
Proline	8·95 (2)	13·02	10·98	1·131	9·43 (3)	14·42	12·16	1·252	9·9	15·1
Hydroxyproline	8·22 (6)	13·62	11·75	1·039	7·37 (6)	12·83	11·07	0·978	8·0	14·0
Arginine	15·16 (2)	8·34	7·48	0·479	14·22 (3)	8·22	7·37	0·472	15·3	8·8
Histidine	0·05 (2)	0·29	0·26	0·019	1·02 (4)	0·70	0·62	0·045	1·2	0·8
Hydroxylysine	0·88 (2)	0·90	0·80	0·055	0·93 (2)	1·00	0·89	0·062	1·2	1·3
*allo*Hydroxylysine‡	0·38 (2)	0·39	0·34	0·024	0·14 (2)	0·15	0·13	0·008	—	—
Lysine	3·87 (4)	3·57	3·13	0·244	4·08 (6)	3·96	3·47	0·271	4·7	4·5
Aspartic acid	3·60 (3)	6·05	5·23	0·454	3·93 (3)	6·95	6·01	0·522	3·6	6·3
Glutamic acid	5·93 (4)	11·02	9·69	0·749	5·69 (3)	11·16	9·75	0·756	5·8	11·3
Amide	2·92 (3)	0·52	—	(0·369)	3·50 (4)	0·66	—	(0·465)	3·5	0·66
Hexosamine	—	0·01	—	—	—	0·05	—	—	—	—
Total	97·30	—	91·56	10·114§	96·97	—	95·97	10·56§	99·0	—
Average residue weight:										
By summation	—	—	90·5	—	—	90·9	—	—	—	92·6
By N-distribution	—	—	95·5	—	—	91·2	—	—	—	92·6

* Bowes & Kenten (1948*a*). † Tristram (1949).
‡ Not identified, but assumed to be *allo*hydroxylysine for purposes of calculation.
§ Amide excluded.
Figures in brackets indicate number of determinations made.

The present values for collagen in some instances differ slightly from those reported earlier. The values for all the basic amino acids, for tyrosine, valine, and to a lesser extent proline, are lower than the earlier values, while the values for serine, aspartic acid and alanine are rather higher. The lower value for tyrosine agrees with independent determinations made on both acid and alkaline hydrolysates by a modification of Lugg's method (Lugg, 1937, 1938*b*), viz. tyrosine N as percentage total N, 0·42. The lower value for lysine

higher value now obtained for aspartic acid substantiates this view. Valine is one of the amino acids for which the Moore & Stein method was found to give the least reproducible results and the older value is, therefore, probably to be preferred. This also applies to hydroxylysine and histidine, where overlapping of the peaks made evaluation difficult. The position of the histidine peak was determined by the addition of histidine to a hydrolysate in one run. A small amount of ninhydrin-reacting material was eluted from the 20-cm. resin

column between hydroxylysine and histidine. In view of the separation of hydroxylysine and *allo*-hydroxylysine reported by Piez (1954), it is probable that this small peak represents *allo*hydroxylysine formed by racemization during hydrolysis. A similar resolution of the hydroxylysine–histidine peak with gelatin hydrolysates is reported by Hamilton & Anderson (1954).

The values given for glycine and hydroxyproline are the first to be determined on this collagen, the previously reported values having been selected from the various determinations made by earlier workers.

The total nitrogen accounted for is 97·1 % and the sum of the amino acid residues is 96·0. The average residue weight by summation is 90·9, and by calculation from the nitrogen distribution 91·2. These values are slightly lower than those previously calculated (Bowes & Kenten, 1948*a*).

The composition of the citrate-soluble collagen of calf skin is essentially similar to that of adult ox-hide collagen, though there are a number of small differences. In view of the lower nitrogen content compared with collagen, these differences are best compared on the basis of amino acid nitrogen as percentage total nitrogen. The amide, tyrosine and histidine contents of the citrate-soluble protein are definitely lower, and the leucine, isoleucine, and possibly the aspartic acid contents are slightly lower than in collagen, while the hydroxyproline content is higher. The ninhydrin-positive material eluted between hydroxylysine and histidine, and assumed to be *allo*hydroxylysine, is three times as great as in collagen.

The total nitrogen accounted for is 97·3 % but the sum of the amino acid residues is only 91·6. This, together with the lower nitrogen content compared with collagen, suggests the presence of some non-protein constituent.

The results of experiments on the reaction of collagen and the citrate-soluble collagen with 1-fluoro-2:4-dinitrobenzene have already been reported (Bowes & Moss, 1953). On the basis of the rather lower lysine content now found for collagen the percentage recovery of lysine as εDNP-lysine from the DNP-protein is increased from 55 to 63 % and is of the same order as that found for the citrate-soluble collagen, namely 69 %. Conversion of the citrate-soluble collagen into gelatin by heating to 40° for 5–10 min. resulted in an increase in the number of free α-amino groups available to FDNB (Table 5). These were similar to those found in commercial gelatins (Courts, 1954), except that the proportion of glycine was much smaller. From the amounts present the number-average molecular weight was found to be 160000.

Table 5. *Terminal residues in citrate-soluble collagen*

	Citrate-soluble collagen	Gelatin from citrate-soluble collagen
	(m-moles/100 g. DNP-protein)	
Aspartic acid	0·06	0·14
Alanine	0·04	0·20
Glycine	—	0·14
Glutamic acid	—	0·17
Serine	—	Trace
Threonine	—	Trace

Sulphur distribution of collagen

The sulphur distribution of the ox-hide collagen is given in Table 6. Values previously reported by Baernstein (1932) and Beach & Teague (1942) for total sulphur and ester sulphate in gelatin are rather higher, probably owing to the presence of impurities arising from contamination with hair.

An appreciable amount of the total sulphur is not accounted for; though some of this deficit may be due to cystine present as an impurity, it is probable that the value for sulphate sulphur is low owing to the difficulties of determining such small amounts.

Assuming that this sulphate sulphur is all derived from chondroitin sulphate, the amount of this polysaccharide present would be of the order 0·4 %, corresponding to a galactosamine content of 0·17 %.

DISCUSSION

The question of whether the protein extracted from skin by citrate buffers is a precursor of collagen, and represents a definite entity, or whether it represents the solution of the collagen fibres as a whole has been the subject of some discussion (Harkness, Marko, Muir & Neuberger, 1953, 1954; Neuberger, Randall & others, 1953). As far as the present experiment is concerned, the extraction of

Table 6. *Sulphur distribution of gelatin and collagen*

Results are expressed as g./100 g. of moisture and ash-free protein.

	Total S	Methionine S	Cystine and cysteine S	Sulphate S	S not accounted for
Gelatin (Baernstein, 1932)	0·47	0·21	0·13	0·10	0·03
Gelatin (Beach & Teague, 1942)	0·411	0·175	0·005	0·207	0·024
Ox-hide collagen	0·30	0·18	—	0·03	0·09

calf skin with citrate buffer appears to involve, in the main, the removal of a specific soluble fraction rather than continued slow solution of the skin substance as a whole, the proportion of nitrogen extracted gradually decreasing until it reaches a low constant value. The readiness with which the citrate-soluble protein is precipitated by the addition of sodium chloride to the extract, and the fibrous nature of the precipitate formed suggests that it is more highly orientated than gelatin. On the other hand, its ready conversion into gelatin indicates that it is less heat-stable than collagen. About two-thirds of the total protein extracted by citrate buffer is precipitated by salt and is of a collagenous type (the procollagen of Orekhovich). The remaining third may partly consist of albumins and globulins, but subsequent experiments on the more exhaustive extraction of tendon indicate that a protein fraction differing from these is also present (see p.146).

The amino acid composition of ox-hide collagen as determined by the Moore & Stein (1951) technique differs slightly from that reported for the same sample in 1948 (Bowes & Kenten, 1948*a*). The recovery of nitrogen was not quite complete; this was probably due to overall losses of amino acids, since it was accompanied by a corresponding deficiency in the sum of the amino acid residues.

With the citrate-soluble protein the amino acids found account for 97·3% of the total nitrogen, while the sum of the weights of the amino acid residues is only 91·6 g. Thus, even if all the nitrogen unaccounted for was present as amino acids of high molecular weight, the total of the residue weights (g.) would still fall appreciably short of a hundred. This, together with the low nitrogen content compared with collagen, suggests the possible presence of about 4–5% of some constituent of low nitrogen content. No independent evidence of such a constituent has been found, however. Determination of reducing sugars and hexosamine, as well as paper chromatography and electrophoresis, indicate the presence of only very small amounts of hexosamine, galactose, glucose and mannose amounting in all to less than 0·1%, so that no appreciable amount of polysaccharide or carbohydrate is present (see Appendix).

The amino acid composition of the citrate-soluble protein differs in some respects from that reported by Chernikov (1949) and Orekhovich (1952) for procollagen. The histidine content is much lower and the sum of the proline and hydroxyproline is appreciably higher.

In view of the low nitrogen content of the citrate-soluble collagen, comparison of its composition with that of adult collagen was made on the basis of amino acid nitrogen as a percentage of the total nitrogen. It is possible that any differences observed in the present investigation are due to variations in age and breed. In view, however, of the small variations in the composition of collagen from widely different sources reported by Neuman (1949) this possibility, though not completely excluded, appears to be small. The composition of the two proteins is essentially the same, but there are a few definite differences which suggest that the adult collagen is associated with a protein constituent which is relatively rich in amide nitrogen, tyrosine, histidine and, to a lesser extent, leucine, isoleucine and possibly aspartic acid, and low in hydroxyproline compared with the citrate-soluble collagen. Evidence obtained from subsequent experiments on the exhaustive extraction of tendon suggest that a protein fraction of similar composition is extracted to some extent by citrate buffers, together with the collagenous protein, and probably represents part of the protein remaining in solution after the addition of sodium chloride (see p.146). The relatively high hexosamine/nitrogen ratio of the solution after precipitation with sodium chloride suggests that this soluble protein fraction may be associated with polysaccharide.

Other workers have also found evidence for the presence of such a constituent in collagenous tissue. Eastoe & Eastoe (1954) suggest its presence in bone collagen, Consden, Glynn & Stanier (1953) in connective tissue, and Harkness *et al.* (1954) and Consden & Bird (1954) in rabbit skin. The small amounts of sugars present in collagenous tissue probably arise from this mucopolysaccharide, rather than from the collagen itself. The almost complete absence of sugars in the citrate-soluble protein would appear to substantiate this view. The sugars associated with ox-hide collagen are the same as those identified by Consden *et al.* (1953) in connective tissue and by Eastoe & Eastoe (1954) in bone collagen, though they differ in their relative proportions and are much smaller in amount (see Appendix). It is reasonable to suppose that skin with its closer fibrous structure contains less mucopolysaccharide than the looser types of collagenous tissue. It is possible that this protein–polysaccharide fraction represents the main constituent of the ground substance. It appears to be removed from collagenous tissue by alkaline treatment (Bowes *et al.* 1953; Consden & Bird, 1954; Eastoe & Eastoe, 1954), and possibly by dilute acids (Bowes *et al.* 1953; this paper, see above). This removal appears to run parallel with reduced stability of the collagen, prolonged treatment in alkaline solutions facilitating the conversion of collagen into gelatin, and treatment in dilute acetic acid favouring dispersion of the collagen. The ready conversion of the citrate-soluble collagen into gelatin also suggests that separation from the non-collagenous fraction reduces stability.

The nature of this non-collagenous protein fraction and its relationship with the citrate-soluble collagen and the insoluble collagen of adult skin is being further investigated.

SUMMARY

1. Calf skin has been extracted with dilute citrate buffer and the soluble collagen precipitated with sodium chloride.

2. The amino acid composition of the citrate-soluble collagen as determined by the Moore & Stein (1951) technique was essentially the same as that of ox-hide collagen. The citrate-soluble collagen contained less amide N, tyrosine and histidine and rather more hydroxyproline than the ox-hide collagen. Its lysine, leucine, isoleucine and aspartic acid contents were also slightly lower.

The values obtained for some of the amino acids in ox hide differed slightly from those reported in 1948 (Bowes & Kenten, 1948*a*) for the same sample.

3. Reducing sugar and hexosamine determinations indicated that neither hide collagen nor the citrate-soluble collagen contained any appreciable amount of polysaccharide. The ratio of hexosamine to nitrogen in the citrate extract after precipitation of the collagenous protein suggested the presence of mucopolysaccharide.

4. Aspartic acid and alanine were detected as free terminal residues in the citrate-soluble protein. After conversion into gelatin by heating to 40° a few additional terminal residues were detected.

5. From consideration of the differences in composition between the hide collagen and the citrate-soluble protein it is suggested that the former is associated in some way with a protein constituent which is relatively rich in amide nitrogen, tyrosine, histidine and certain other amino acids, and relatively low in hydroxyproline. Other evidence for the presence of such a constituent in collagenous tissue is cited, and its possible significance with regard to the stability of collagen discussed.

The authors are indebted to Mr P. R. Sanders of British Gelatine and Glue Research Association for determination of the rigidity of the gelatin obtained from the citrate-soluble protein, and to the Director and Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

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APPENDIX

The Carbohydrate of Collagen

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The presence of up to 1 % of sugars in collagen has been suggested by several workers, notably Grassmann & Schleich (1935) and Beek (1941), but none of these workers definitely established their identity. More recently, Glegg, Eidinger & Leblond (1953) have reported the identification by paper chromatography of glucose, galactose, mannose, fucose and a trace of ribose in reticulin and tendon collagen, whilst Consden, Glynn & Stanier (1953), using a combination of paper electrophoresis and paper chromatography, have found glucose, mannose, galactose and glucosamine, the last two predominating, in subcutaneous tissue.

Preliminary attempts to detect sugars in purified skin collagen by these methods were unsuccessful. This failure was attributed to the very low concentrations present and to considerable interference from the high amino acid and peptide content of the hydrolysates.

A number of methods used for the determination of sugars have been applied to collagen. Some estimates made of the interference due to protein hydrolytic products, which are of general interest in connexion with the determination of sugars in other proteins, have been recorded.

EXPERIMENTAL

Materials. The collagenous materials used were similar to those described in the main part of this paper.

Hydrolysis. The protein was hydrolysed with 2 N-HCl for 16 hr. in a sealed tube at 105°.

Reducing sugars. The method of Somogyi (1945) and the method of Hagedorn & Jensen (1923) as modified by Lampitt, Fuller, Goldenberg & Green (1947) were used. A modification of the anthrone colorimetric method (see Scott & Melvin, 1953) was also used. To each of several tubes 6 ml. of 0·15 % (w/v) anthrone in 87 % (v/v) H_2SO_4 were added. After cooling in ice for 5 min., 1 ml. of unknown or standard containing up to 200 μg. of glucose or its equivalent was added slowly to form a top layer. After cooling for a further few min., the contents of the tubes were mixed by inversion. The stoppered tubes were then transferred to a water bath at 80° for 15 min. After cooling, the absorption was measured on a Hilger 'Uvispek' spectrophotometer at 625 mμ. Scott & Melvin (1953) report that the anthrone colour is increased in the presence of chloride ions which may be present in hydrolysates. In the present work it was found that this increase was virtually independent of chloride concentration above 0·1 N and was of the order of 9 %.

Hexosamine. This was determined by the method of Elson & Morgan (1933) as modified by Blix (1948). Following the suggestion made by Schloss (1951), readings were also made at 515 mμ. after 20 hr. incubation at 30° using a Hilger 'Uvispek' spectrophotometer. When the determinations were made on the 1 ml. fractions eluted from a chromatographic column the reaction was carried out in the tubes used for the collection of the samples with appropriate adjustments to the volumes of reagents added.

Paper chromatography. Two-dimensional chromatograms were run on Whatman no. 2 paper, 24 in. square. The systems phenol–water and *n*-butanol–water were found to be a suitable combination of solvents for satisfactory separation of glucose, galactose and mannose when each was run the full length of the paper. The papers were sprayed with aniline hydrogen phthalate reagent (Partridge, 1949), and, after heating for 5 min., examined in ultraviolet light.

Chromatograph technique. Hexosamines were separated from other material present in the hydrolysates, on a column of Dowex-50 1 × 23 cm. high, similar to that used by Moore & Stein (1951) for the separation of basic amino acids (see also Eastoe, 1954). Under these conditions glucosamine emerged after 84 ml. Determination by ninhydrin and by the modified Elson & Morgan (1933) procedure gave the same values, indicating that the glucosamine emerged free from peptides and amino acids. Thus, either method may be used for the determination. Using a larger column (2 cm. diam. × 20 cm.) of Dowex-50 equilibrated with 0·05 N-HCl and the same acid as developer, it was found that hexoses and uronic acids were eluted together and free from amino acids, etc., after about 20 ml. of acid had passed through the column (cf. Gardell, 1953).

RESULTS AND DISCUSSION

Attempts to determine reducing sugars directly on a collagen hydrolysate gave results depending on the method used (Table 1, columns 1 and 2). Trials with a synthetic hydrolysate containing the correct proportions of all the amino acids, with the exception of hydroxylysine, which have been identified in collagen, indicated that the interference due to these was considerable with the Hagedorn & Jensen (1923) method, but was relatively slight in

Table 1. *Reducing sugars in collagen and citrate-soluble collagen (CS-collagen)*

The results are expressed as mg. glucose/100 mg. protein.

Method	Uncorrected: Collagen (1)	Uncorrected: CS-collagen (2)	Synthetic hydrolysate (3)	Corrected for the contribution of amino acids (col. 3): Collagen (4)	Corrected for the contribution of amino acids (col. 3): CS-collagen (5)	Neutral sugars + uronic acids after separation on Dowex-50: Collagen (6)	Neutral sugars + uronic acids after separation on Dowex-50: CS-collagen (7)
Hagedorn &Jensen (1923)	2·98	2·40	2·31	0·67	—	0·27	—
Somogyi (1945)	0·32	0·19	0·04	0·28	0·15	0·27	—
Anthrone	0·33	0·43	0·04	0·29	0·39	0·26	0·01–03

Table 2. *Hexosamine content of collagens*

The results are expressed as mg. glucosamine/100 mg. protein.

	Collagen	Citrate-soluble collagen	Synthetic hydrolysate
Uncorrected values	0·30	0·15	0·07
Glucosamine equivalent of unacetylated blanks	0·11	0·10	0·02
Values corrected for blanks	0·19	0·05	0·05
After separation on Dowex-50	0·05	0·01	—

the Somogyi (1945) and anthrone methods (Table 1, column 3). The amino acids chiefly responsible for the high values in the Hagedorn & Jensen method were tyrosine, methionine, proline and hydroxyproline, which gave 76, 42, 3 and 2 %, respectively, of the values obtained for the same weight of glucose. After correction for the contribution of the amino acids (Table 1, columns 4 and 5) the Hagedorn & Jensen method still gives higher values for collagen than the other two methods. The correction is not valid for the citrate-soluble collagen owing to its much lower tyrosine content; if, however, allowance is made for this, a value of the same order as that found by the Somogyi method is obtained. The determination of hexosamine is also subject to interference by protein hydrolytic products. Colour contributed by hydroxyproline under the present conditions was 0·1 % of that of an equal weight of glucosamine. In collagen this contribution would represent about 0·015 % of glucosamine. Colour may also be contributed by amino acid–sugar reactions (Vasseur & Immers, 1949; Stary, Yenson, Lisie & Bilen, 1951; Horowitz, Ikawa & Fling, 1950) and is particularly important if small amounts of glucosamine are to be determined.

It has been suggested (Anastassiadis & Common, 1953) that allowance can be made for this interference by the inclusion of blanks of the hydrolysate treated exactly as the test solution except that sodium carbonate containing no acetylacetone is used. With both collagen and citrate-soluble collagen the blanks obtained in this way were relatively large (see Table 2). This does not, however, give an entirely satisfactory blank since the values obtained for the synthetic hydrolysate showed that acetylation increased the contribution due to amino acids, and Vasseur & Immers (1949) state that the same is also true of the amino acid–sugar reaction products. Development of the colour for 24 hr. as recommended by Schloss (1951) gave similar results.

As it appears from the above that direct determinations of reducing sugars and hexosamines in collagen hydrolysates give misleading results, separation of these substances from other protein hydrolytic products using Dowex-50 was investigated (see 'Methods').

Hydrolysate equivalent to up to 1 g. protein was put on the larger-diameter column and developed with 0·05N-HCl, 1 ml. fractions being collected. Hexoses were determined by the anthrone method on 0·2 ml. portions of the fractions. Those containing anthrone positive material were then bulked and the reducing power towards the Somogyi (1945) and Hagedorn & Jensen (1923) reagents determined (Table 1, column 6). The values obtained by the three methods agree. Since galacturonic acid gives 5 % of the colour yield of an equal weight of glucose with the anthrone reagent, 14 % of the reducing power towards the Hagedorn & Jensen reagent and 90 % towards the Somogyi reagent, it may be concluded that the amount of uronic acid present is very small. The remaining eluate was evaporated to dryness and paper chromatograms were run. Glucose and galactose in approximately equivalent quantities and a smaller amount of mannose were found.

A similar procedure in the case of citrate-soluble collagen showed the presence of glucose, galactose and mannose, the spots being all of approximately the same size and intensity on the paper chromatogram. In neither material was uronic acid detected.

For the separation of hexosamine, samples of hydrolysate equivalent to about 100 mg. protein were put on the smaller column. 1 ml. fractions were collected and hexosamine was determined by the modified Elson & Morgan procedure. The results

are given in Table 2. The amount of hexosamine found in the ox-hide collagen by this method was even smaller than the corrected value obtained directly on the hydrolysate, and the amount found in the citrate-soluble collagen was negligible.

The sugars and sugar derivatives which have been identified in ox-hide collagen are the same as those found in other collagenous tissues, e.g. tendon, but the relative proportions are different and their total amount is less, especially in the citrate-soluble collagen. The negligible amount of hexosamine and the very small and variable amounts of hexoses found in the soluble protein suggest that these may be present as impurities. This is contrary to the views of Küntzel (1954) and of Grassmann, Endres & Steber (1954) who have found appreciable amounts of hexoses and amino sugars, respectively, which they consider to be an integral part of the citrate-soluble collagen molecule.

SUMMARY

1. Some methods used for the determination of sugars have been examined. Their application to complete protein hydrolysates is discussed.

2. The carbohydrate content of ox-hide collagen and citrate-soluble collagen has been determined.

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COLLAGEN AND THE MORE SOLUBLE CONSTITUENTS OF SKIN*

By J. H. Bowes, R. G. Elliott, and J. A. Moss.

SUMMARY

The chief protein constituent of skin is collagen, but experiments on extraction with dilute acid and alkaline solutions indicate the presence of a number of different protein fractions differing in solubility. The methods used for the determination of the amino acid composition and terminal amino groups of such protein fractions are briefly described.

The composition of ox hide collagen has been redetermined by the method of Moore and Stein, and some slight differences from previous analyses are pointed out. Attention is drawn to the peculiarities of the amino acid composition of collagen. No terminal amino groups have been detected in the original collagen but after relatively mild treatments a number become available for reaction.

A collagenous protein has been extracted from calf skin with citrate buffer—the procollagen of Orekhovich—and some of its properties are described. The amino acid composition of the extracted protein is essentially the same as that of the ox-hide collagen, but certain small differences suggest that the ox-hide collagen is associated with a protein relatively rich in tyrosine, histidine, leucine, and amide-nitrogen, and containing little or no hydroxyproline.

Further experiments on the extraction of calf skin with citrate buffer, dilute acetic acid, and alkali indicate the presence of one or possibly two non-collagenous protein constituents closely associated with the collagen.

The significance of the various soluble protein fractions in relation to the pretanning processes is discussed.

The composition of collagen and the other constitutents of skin, and how they are affected by the early processes of leather manufacture, has always been of interest to the leather chemist, and numerous papers on the subject have appeared in the literature. In recent years there has been an increasing interest in the biological and medical fields concerning the more soluble constituents of collagenous tissue and the part which they play in the formation, metabolism and diseases of such tissue. In leather chemistry also, the import-

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ance of these constituents in determining the properties of the skin has been more fully realised. The present paper summarises some of the work which has been carried out in these laboratories during the past five years on the composition of skin collagen and the proteins extracted from skin by the action of dilute acid and alkaline solutions.

Fresh skin consists largely of water; there is some 30–35% protein, excluding the hair, 1–10% grease, or even more in the case of sheepskins, and a small amount of carbohydrate (Fig. 1.). There do not appear to be any figures available for the carbohydrate content of fresh skin, but judging from the low values obtained for the extracted proteins and the remaining insoluble collagen, it cannot amount to much more than 0·5% of the wet weight. Of the 30–35% protein, about 4–6% is made up of the interfibrillary proteins, such as albumins and globulins of the tissue fluids, about 0·5–1·0% represents the epidermis and there are small amounts of muscle protein, elastin and reticulin. The insoluble residue of protein obtained after autoclaving, (0·6%) may be the remains of these or may be an additional constituent. By far the greater part of the protein is collagen (90–95%), but it is now becoming increasingly clear that what has generally been referred to as collagen is not homogeneous and can be divided into a number of different fractions. The term collagen will, as far as possible, be restricted to the protein which is the chief constituent of the collagen fibres and which so far has probably never been obtained pure.

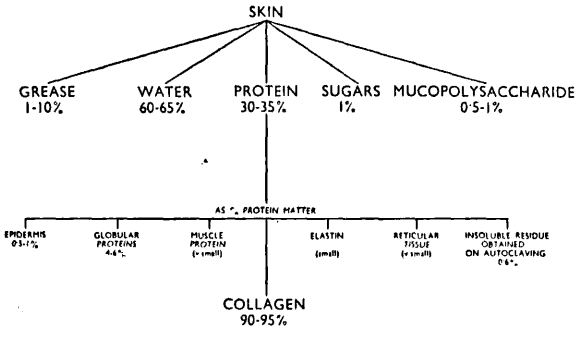

FIG. 1.
The constituents of fresh skin.

The partial dissolution of collagen fibres in dilute solutions of weak acids, such as formic and acetic has been known for many years[1,2,3,4], and more recently, interest in this subject has been revived by the work of Orekhovich[5,6] and his colleagues in Russia. They report the extraction of a soluble collagenous type protein from the skins of animals using dilute citrate buffers. This protein was precipitated by salts or by dialysis and the fibrous precipitate formed had the characteristic x-ray diffraction and electron microscopic appearance of collagen fibres. They found that more of this collagenous protein could be extracted from the skin of young animals than from old and

suggested that it might be a soluble precursor of collagen and called it procollagen. The isolation of this protein has been confirmed by workers in this country[7,8] and in the States[9], but the view that it is the true precursor of collagen is not generally accepted. Harkness, Marko, Muir, and Neuberger[7], and Schmitt, Gross and Highberger[10,11] have isolated another collagenous fraction which is soluble in phosphate buffers at pH 9·0 and in dilute sodium chloride solutions. On the basis of radioactive isotope experiments, Harkness *et al*[7] consider that this is the more likely precursor. On solution in citrate buffer, this fraction is apparently converted into a protein resembling procollagen and is no longer soluble in alkali[7]. On heating to 37° for a few minutes it forms a fibrous precipitate showing the characteristic striations of normal collagen fibres under the electron microscope[11].

Thus, the collagen of skin can be divided into at least three different fractions of varying solubility. Evidence is also accumulating for the presence of at least one or possibly two protein constituents closely associated with the collagenous type of protein[7,12,13].

Experimental Methods.

Determination of Amino Acid Composition.

Until recently, the determination of the amino acid composition of a protein was a very long and tedious operation requiring several grams of material. Although it was possible to distinguish broad differences between the composition of proteins by simpler means, it was impossible, for example, to know whether gelatin had the same composition as collagen or whether protein extracted from skin represented a specific fraction of the collagen molecule or an entirely different entity, without being involved in a very large amount of time-consuming analytical work requiring considerable amounts of protein.

In recent years, however, methods have been developed which enable a complete amino acid analysis to be carried out in a little over a week, using only a few milligrams of protein. These[14,15], and another technique[16] to which reference will be made later, have their origin in the work of Martin and Synge[17] on partition chromatography carried out in the laboratories of the Wool Industries Research Association, and which has found a wide application in a number of fields.

The method used in the present investigation for the determination of the amino acid composition was that developed by Moore and Stein[15], which involves separation of the amino acids on an ion exchange resin followed by colorimetric determination with ninhydrin. This method is represented diagrammatically in Fig. 2. After hydrolysis with hydrochloric acid in the usual way, a sample of hydrolysate, containing the equivalent of 3–8 mg protein is placed on a column of ion exchange resin, 100 cm long by 0·9 cm in diameter. The resin used is Dowex 50, a sulphonated polystyrene resin of approximately 8% crosslinking and the separation of the amino acids depends on their pk values, their molecular size and shape, and on the influence of side chain groups such as hydroxyl. By using a series of buffers of increasing

pH and eluting at three different temperatures, 35, 45 and 75°C it is possible to obtain separation of all the amino acids, each being washed off the column successively.

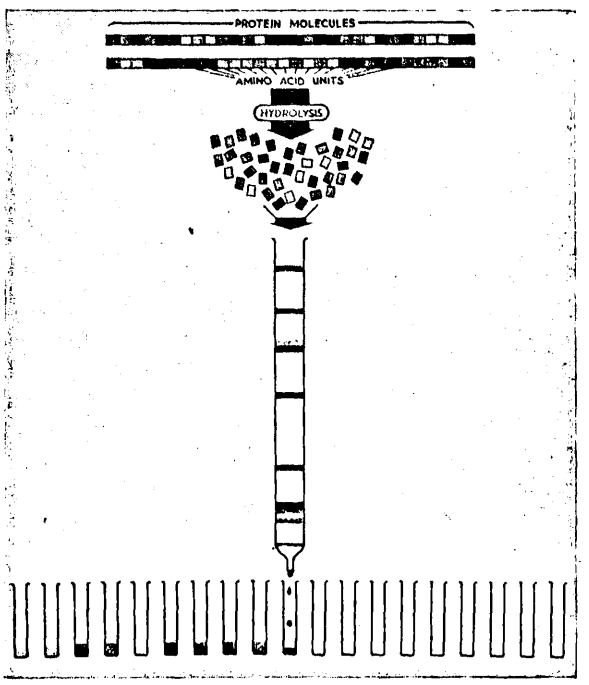

FIG. 2.
Diagrammatical representation of the chromatographic separation of amino acids.

The eluate from the column is collected in 1 ml. fractions, three to four being collected every hour. As some six hundred or more fractions are involved it is necessary to have an automatic device for this. There are now a number of such fraction collectors commercially available, but at the time this work was started this was not so. An apparatus was, therefore, constructed in the laboratory. The eluate from the column flows into a collecting syphon which when full delivers into a series of test tubes held in a circular collecting disc. The passage of the liquid down the side arm of the syphon operates a capacitance relay (a Tektor Laboratory Model by Fielden) which starts a motor and rotates the disc so that the next tube is brought beneath the syphon.

The amino acid in each fraction is then determined colorimetrically with ninhydrin—this gives a purplish-blue colour with all the amino acids, a reddish colour with hydroxyproline and a yellow colour with proline. From the intensity of the colour obtained the amount present in each fraction is obtained by reference to a standard curve. The amounts of amino acid involved are very small, of the order of 0·005 to 0·05 mg.

The sort of separation of the amino acids obtained is illustrated in Fig. 3, in which the amino acid content of each fraction is plotted against the number of that fraction. There is very little overlap of the peaks, except with hydroxyproline and aspartic acid. This, however, is not serious, the orange colour due to hydroxyproline does not appreciably interfere with the measurement of the blue colour due to aspartic acid, and as the ninhydrin method for hydroxyproline is, in any case, not very sensitive, this amino acid was generally determined independently by the method of Neuman and Logan[18]. Glycine and alanine, which are present in very high concentrations in collagen, also overlap sometimes and it is usually more convenient to determine these separately using less protein.

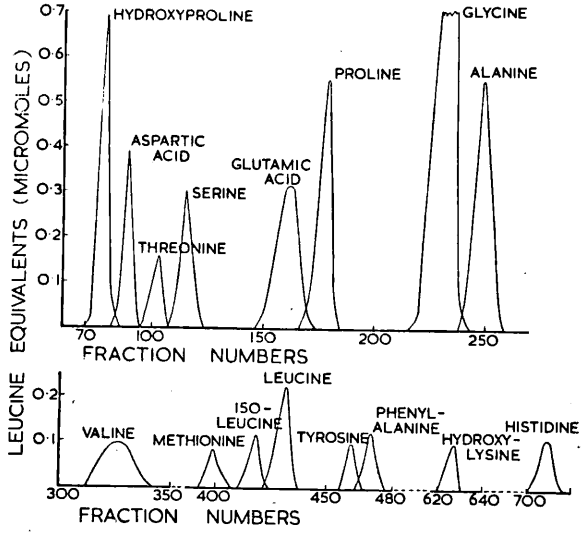

FIG. 3.

Separation of amino acids in a hydrolysate of collagen on 100 c.m. column of Dowex 50. Hydrolysate equivalent to about 4·5 mg protein applied to column. Buffers used for elution fractions 0–260 pH 3·42 at 37·5°C; 260–425 pH 4·25 at 28°C; 425–560 pH 4·25 at 75°C; 561–585 pH 6·0 at 25°C; 586–695 pH 8·3 at 25°C; 696–720 pH 9·2 at 25°C.

There is some loss of the basic amino acids which are eluted from the column last, and in practice these are determined using a shorter column 15–20 cm long and a different series of buffers (see Fig. 4). In this way the bulk of the amino acids is eluted in the first few fractions, followed by the basic amino acids. The two small peaks emerging just before histidine are most probably[19] hydroxylysine and aleo-hydroxylysine respectively[20].

DETERMINATION OF TERMINAL AMINO RESIDUES.

The method developed by Sanger[16] for the determination and identification of the amino acid residues supplying the terminal amino group in proteins and peptides was applied to collagen and modified collagens. The

principle of the method is represented diagrammatically in Fig. 5. The protein or peptide is treated with 1-fluoro-2:4-dinitrobenzene which reacts with the amino groups present. The protein is then hydrolysed, leaving the dinitrophenyl (DNP) group attached to the ε-amino groups of lysine and hydroxylysine and to the α-amino groups of the terminal amino acids. The yellow dinitrophenyl derivatives of the terminal amino acids are then extracted into ether, leaving the water-soluble ε-DNP-lysine and hydroxylysine, and the remaining unreacted amino acids in the water phase.

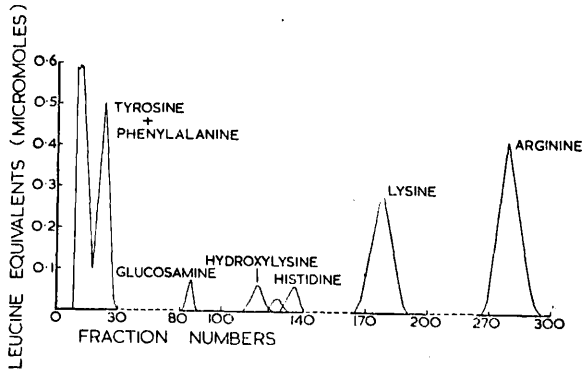

FIG. 4.

Separation of basic amino acids in a hydrolysate of collagen on a 20 cm column of Dowex 50. Hydrolysate equivalent to about 8·5 mg protein applied to column. Buffer used for elution—Fractions 0–30 pH 5·0 at 25°C; 31–140 pH 6·8 at 25°C; 141–300 pH 6·5 at 25°C.

The α-DNP—amino acids are then separated chromatographically on silica gel and determined colorimetrically. The method has been applied to collagen and to collagen treated in various ways to determine the extent of the breakdown which takes place and where it occurs. It has also been used extensively by other workers in studies on the degradation of gelatin by acid, alkali, heat and enzymes[21,22,23].

Experimental Results.

AMINO ACID COMPOSITION OF COLLAGEN.

The amino acid composition of ox-hide collagen has been redetermined[18] using material from the same sample as that for which analyses were published in 1948[24]. This sample was prepared from the middle split of a fresh ox-hide, and the interfibrillary proteins were removed by drumming in several changes of 5% sodium chloride solution. By removing the grain layer which contains hair roots, sebaceous and sweat glands, and most of the elastin and reticulin, it was hoped that complications due to the presence of extraneous matter would be small, and that apart from a small amount of elastin and reticulin, the material obtained would be what is generally referred to as collagen.

The amino acid composition of this collagen sample determined by the method of Moore and Stein and expressed as g per 100 g and as amino acid nitrogen as per cent of the total nitrogen is shown in Table I.

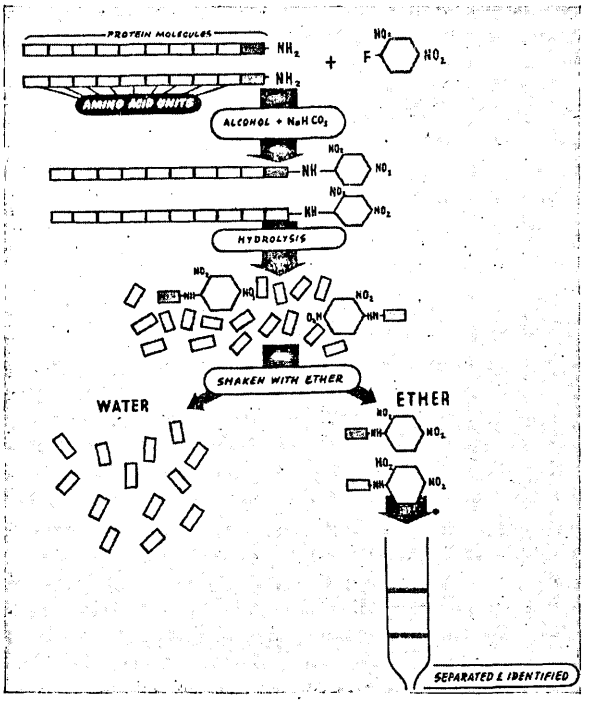

FIG. 5.
Diagrammatic representation of the fluorodinitrobenzene technique for the determination of α-terminal amino groups.

There are a few small differences from the analysis which was reported some years ago :— the values for lysine, tyrosine, and to a lesser extent proline are lower ; and the values for serine alanine and aspartic acid are higher.

The lower value for lysine now reported agrees better with determinations of free amino groups, and that for tyrosine agrees with independent determinations made by the methods of Lugg[25] and Udenfriend and Cooper[26]. The higher value for serine and aspartic acid are confirmed by the results obtained by Eastoe[27] on gelatin obtained from different sources. The values for glycine and proline are the first to be determined on this particular batch of collagen, the values previously reported having been selected from the literature.

Ninety-seven per cent of the total-nitrogen has been accounted for; the deficiency of 3% is probably due to small overall losses of amino acids.

There are a few peculiarities regarding the amino acid composition of collagen which are of interest. First of all, the very high content of proline and hydroxyproline; the proline content is higher than that of any other protein, while hydroxyproline has not been found in any other protein, except elastin, in which it only occurs to the extent of about 1%. The hydroxyproline content of a tissue is, therefore, a useful guide to its collagen content, and has, in fact, been used by a number of workers for the determination of collagen.

Collagen is also the only protein which contains an appreciable amount of hydroxylysine. Secondly, the low content of aromatic amino acids; tryptophan is absent and the tyrosine content is very low. In fact, collagen is a very unbalanced protein, and this is well illustrated by the Moore and Stein separation curves (Fig. 3 and 4). Approximately half its weight is made up of four units, gycine and alanine, and proline and hydroxyproline, i.e. two of the smallest units and two of the more bulky units; rather more than another quarter is made up by the acidic and basic amino acids, leaving less than a quarter for the nine remaining neutral amino acids.

TERMINAL AMINO GROUPS IN COLLAGEN AND MODIFIED COLLAGEN[28].

No terminal amino groups were detected in collagen using the Sanger method, and Grassmann and Hormann[29] using the same technique confirm this. The apparent absence of a terminal amino group may be due to the molecule being very large, greater than 4 or 5 million; the molecule may be cyclic, though this does not fit in with the general ideas on the molecular structure of collagen; or the terminal group may be inaccessible.

There is the possibility that end groups may be masked by polysaccharide and treatment with testicular hyaluronidase was, therefore, carried out to see whether degradation of hyaluronic acid and chondroitin sulphate led to the

TABLE I.

COMPOSITION OF COLLAGEN AND CITRATE-SOLUBLE COLLAGEN.

	g. per 100 g. Protein		N. as % Total-N.	
	Collagen	Citrate-Soluble Collagen	Collagen	Citrate-Soluble Collagen
Total-N	*18·60*	*17·70*	—	—
Amino-N	0·46	0·49	2·5	2·7
Glycine	26·6	26·1	26·7	27·5
Alanine	10·3	10·0	8·7	8·8
Leucine	*3·7*	*3·2*	*2·1*	*1·9*
Isoleucine	*1·9*	*1·4*	*1·1*	*0·8*
Valine	2·5	2·3	1·6	1·5
Phenylalaline	2·4	2·0	1·1	1·0
Tyrosine	*1·0*	*0·5*	*0·4*	*0·2*
Tryptophan	—	—	—	—
Serine	4·3	4·2	3·1	3·2
Threonine	2·3	2·2	1·4	1·5
Cystine	—	—	—	—
Methionine	1·0	0·8	0·5	0·4
Proline	14·4	13·0	9·4	8·9
Hydroxyproline ...	*12·8*	*13·6*	*7·4*	*8·2*
Arginine	8·2	8·3	14·2	15·2
Histidine	*0·7*	*0·3*	*1·0*	*0·4*
Hydroxylysine	1·2	1·3	1·1	1·3
Lysine	4·0	3·6	4·1	3·9
Aspartic Acid	*7·0*	*6·0*	*3·9*	*3·6*
Glutamic Acid	11·2	11·0	5·7	5·9
Amide	*0·7*	*0·5*	*3·5*	*2·9*
Hexosamine	0·05	0·01	—	
Reducing Sugars ...	0·26	0·01–0·03		
Total			97·0	97·1

liberation of any terminal residues. Unfortunately, the results were inconclusive, for although small amounts of aspartic acid, alanine, glycine and threonine were found as terminal residues it was not certain whether they definitely arose from the removal of polysaccharide.

Aspartic acid is very readily released as a terminal residue by such mild treatments as heating to 60°C for 5 minutes or treatment in urea so that this may be the terminal residue, if not, then the peptide bond involving this amino group is very labile. After treatment with alkali, aspartic acid, glutamic acid, glycine and alanine were found as terminal residues, and apart from the substitution of phenylalanine by threonine, the same residues were found in a commercial gelatin.

The liberation of a number of end groups during solution in formic acid is of interest in view of the use of this method for obtaining solutions of collagen. The results suggest that the solution obtained in this way more nearly resembles one of gelatin than one of collagen.

Extraction of Citrate-Soluble Collagen.

Having obtained information on the composition of adult ox collagen fibres, consideration was given to the composition of the more soluble collagenous fractions which can be extracted from skin[8].

A calfskin was obtained from the slaughter house immediately after flaying. The skin was shaved, cut into pieces 1 cm square and disintegrated in a Wiley Mill. The disintegrated material was extracted with 0·1M phosphate

TABLE II.

FREE AMINO GROUPS OF COLLAGEN AND MODIFIED COLLAGEN.

	α-amino Groups		ε-amino Groups	
	mmol./100 g	Weight containing one residue	groups reacting mmol./100 g	% reacting
Collagen	No end-groups detected		17	56
Urea-treated collagen				
Aspartic acid ...	0·07	1,400.000	18	59
Heat-shrunk collagen				
Aspartic acid ...	0·15	700.000		
Glutamic acid ...	0·03	3,300,000	15	48
Alkali-treated collagen				
Aspartic acid ...	0·07	1,400.000		
Glutamic acid ...	0·17	600.000		
Glycine	0·42	240,000	17	54
Phenylalanine ...	0·04	2,500.000		
Collagen dissolved in formic acid				
Aspartic acid ...	0·43	230.000		
Glutamic acid ...	0·15	700,000		
Glycine	0·65	150.000	17	56
Alanine	0·30	330.000		
Gelatin (commercial)				
Aspartic acid ...	0·28	350.000		
Glutamic acid ...	0·12	830.000		
Glycine	0·57	180.000	17	56
Threonine	0·14	710.000		

Lysine content of collagen—31 mmol./100g.

buffer at pH 8·6 to remove interfibrillary protein and then four times with 0·12M citrate buffer pH 3·6. The first citrate extract was discarded and the protein from the remainder precipitated by the addition of sodium chloride to a final concentration of 5% w/v. A gelatinous precipitate was formed which was purified by dissolving in slightly acidified water and reprecipitating by dialysis against tap water. On further extraction of a small portion of the macerate, the amount of nitrogen solubilised gradually fell until it reached a low constant value, suggesting the removal of a specific soluble protein fraction rather than continued slow solution of the skin substance as a whole.

From nitrogen determinations on the total extracts it was calculated that a little over 5% of the total protein of the macerate was extracted by the citrate buffer and that a little more than three quarters of this was precipitated by the salt. The identity of the remaining quarter which appeared to be non-collagenous suggested the presence of an additional protein and further experiments which will be described later, confirm this.

PROPERTIES OF CITRATE-SOLUBLE COLLAGEN.

The precipitate formed on the addition of sodium chloride to the citrate extracts was fibrous in appearance and when observed under the microscope the presence of definite fibre-like particles could be seen (Fig. 6).

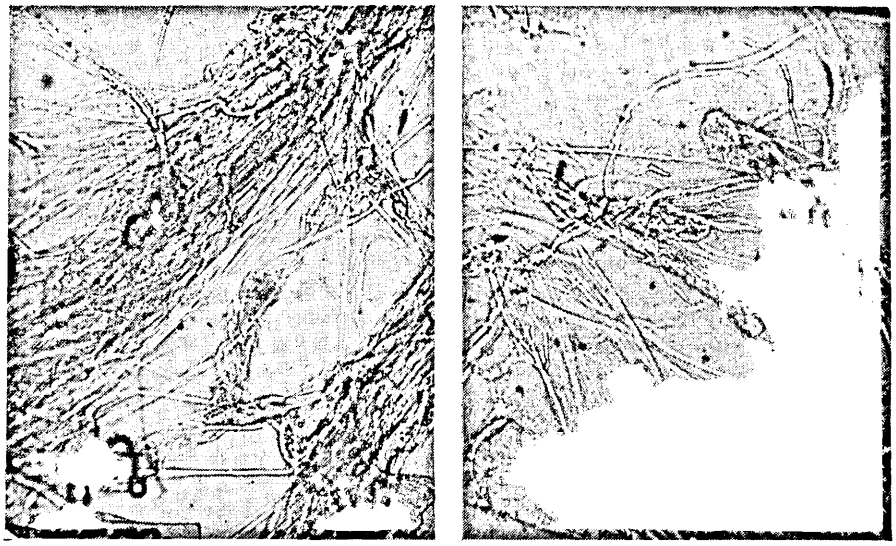

FIG. 6.
Photomicrograph of citrate-soluble collagen after precipitation with sodium chloride.

The staining reactions of this fibrous network were intermediate between those of collagen and reticulin (Table 3).

The protein was soluble in dilute acid buffers to the extent of about 0·5%, solubility decreasing with rise in pH and being negligible above about 6·5. Various workers have shown that it can be precipitated from such solution in a variety of ways giving fibres which, under the electron microscope, may

show striations typical of collagen, more widely spaced striations 2000Å apart or no striations at all. The significance of these is not yet clear, but Gross, Schmitt and Highberger[30,31] in recent papers discuss some of the factors influencing the form in which the soluble collagenous fractions are precipitated and introduce the concept of "tropocollagen". This is considered to be a kinetic unit of collagen, consisting of very thin, elongated particles of length equal to the long spacings observed with collagen. It is suggested that aggregation of these in a particular manner produces fibrous collagen.

TABLE III.

STAINING REACTIONS OF PROCOLLAGEN.

Stain	Reaction
Iodine	Red brown—more deeply staining than collagen
Periodic-acid-Schiffs	Faintly positive like collagen.
Azan and Mallory's Triple Stain	Dense blue like collagen and reticulin.
Haem alum and basic stains	Not stained, resembling collagen and reticulin.
Silver	Purple to black—like reticulin rather than collagen
Toluidine Blue	Not metachromatic.

Measurements of sedimentation rates, light scattering and rates of diffusion made on solutions of acid-soluble collagen from ichthyocol suggest the presence of aggregates of molecular weight about $1 \cdot 67 \times 10^6$, probably consisting of not more than three polypeptide chains[32].

On heating to 40°C for a few minutes and cooling, solutions of the citrate-soluble protein set to a gel, due presumably to conversion to gelatin. The rigidity of such a gel was kindly determined by Mr. P. R. Saunders of the British Gelatine and Glue Research Association and found to be appreciably greater than that of high grade commercial gelatins, determined under the same conditions. In spite of the mildness of the treatment, however, some breaking of the peptide bonds occurred (Table 4.). In the original protein, only very small amounts of aspartic acid and alanine could be detected, but in the gelatin formed from it, both these were increased in amount, and in addition glycine, glutamic acid and traces of serine and threonine were found as terminal residues. The amounts of α-amino groups detected correspond to a number

TABLE IV.

N-TERMINAL RESIDUES IN PROCOLLAGEN

	As extracted	After conversion to Gelatin
	——m-moles/100 g. protein——	
Aspartic acid	0·06	0·14
Alanine	0·04	0·20
Alanine	0·04	0·20
Glutamic Acid	—	0·17
Serine	—	0·14
Threonine	—	trace
Molecular Weight (number average) ...	1,000,000	150,000

average molecular weight of 150,000, a value about twice that found by Courts[21] for alkali processed gelatins using the same method. The terminal residues found in the isolated protein may not be present in the native material, but may have been liberated during preparation.

On the whole, the citrate-soluble collagen seems to have properties midway between collagen and gelatin ; the readiness with which it is precipitated by salts and the fibrous nature of the precipitate suggest that it is more highly orientated than gelatin, while its ready conversion to gelatin suggests that it is less heat stable than collagen.

COMPOSITION OF CITRATE-SOLUBLE COLLAGEN.

Amino acid analysis of the citrate-soluble collagen (Table 2) accounts for 97% of the total nitrogen but the sum of the amino acid residues, which should equal 100, only adds up to 91·6. Even if all the 3% of nitrogen unaccounted for was present in amino acids of high molecular weight, the total of the amino acid residues would still fall appreciably short of 100. This, together with the low nitrogen content of the citrate-soluble collagen compared with collagen, 17·70% compared with 18·60%, suggests the possible presence of 4–5% of some non-protein constituent. No independent evidence of this has been found, however. Determination of reducing sugars and hexosamines indicate the presence of only very small amounts of carbohydrate.

Because of the possibility of a non-protein constituent and the low nitrogen content of the soluble protein, its composition is best compared with that of the ox-hide collagen on the basis of amino acid nitrogen as per cent of the total nitrogen.

The composition of the two proteins is essentially the same, though there are a number of small differences (*see Table 2*). The amide nitrogen, tyrosine and histidine contents of the citrate-soluble protein are lower and the hydroxyproline content is higher, than in collagen. The leucine, isoleucine and aspartic acid contents are also slightly lower than in the ox collagen. These differences suggest that the adult collagen fibres from the ox hide are associated with a protein which is relatively rich in amide, tyrosine, and histidine, and possibly leucine, isoleucine and aspartic acid compared with the citrate-soluble collagen, and which contains little or no hydroxyproline.

It is possible that this constituent is to some extent extracted together with the soluble collagen and represents some of the protein not precipitated by the addition of sodium chloride. Preliminary analysis of the corresponding protein fraction obtained from tendon, after particular care had been taken to remove the albumins and globulins of the tissue fluids, indicated that this had a high amide, tyrosine, leucine and aspartic acid content relative to collagen and a negligible hydroxyproline content.

The citrate solution, after the precipitation of the collagenous protein had a relatively high hexosamine-nitrogen ratio suggesting that the protein remaining in solution is associated with polysaccharide.

Other workers have recently found evidence for the presence of such a constituent in collagenous tissues from other sources and[7,33–35] it was, therefore, decided to investigate it further.

Composition of Protein Fractions Extracted from Calfskin by Dilute Acid and Alkaline Solutions[13].

A fresh calfskin was again taken as the raw material, but in order to have as few complicating factors as possible only the middle layer was used. In this way, contamination with hair roots, sebaceous glands and other extraneous material present in the grain layer was avoided. In order to obtain the middle layer, 1 inch punches from the skin were sectioned on a freezing microtome. The first few sections from the flesh side were discarded, and the following sections collected until the base of the hair roots was reached. The sections were transferred immediately to phosphate buffer solution pH 9·0, and stored below 5°. The skin was also stored in a refrigerator at 4° while awaiting sectioning. About 0·9 g wet weight were obtained from each 1 inch punch and in all about 470 punches were sectioned.

The sectioned calfskin was extracted with 0·1M phosphate buffer to remove albumins and globulins (Fig. 7). The extraction was continued until the nitrogen in the extract became very small, and in all five extractions were made. It was then extracted seven times with citrate buffer pH 3·7 until the nitrogen content of the extract was again very small. The citrate buffer was followed by 0·05M acetic acid adjusted to pH 2·5 with hydrochloric acid, six further extractions being made. The lowering of the pH caused the amount of protein extracted to increase slightly and then after successive extractions to fall again to a low value.

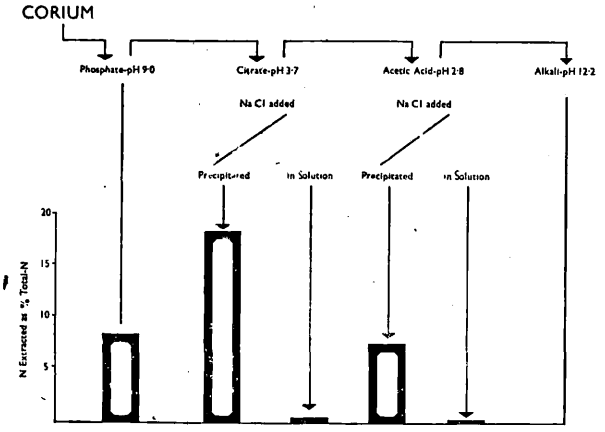

FIG. 7.
Extraction of calf skin with dilute acid and alkaline solutions.

The skin pieces were finally neutralised and extracted with several changes of alkali at about pH 12–12·3.

The amount of nitrogen extracted at the various stages, expressed as a percentage of the total nitrogen, are given in Fig. 7. About 8·5% was extracted by the phosphate buffer, 20% by the citrate buffer, 8% by the dilute acetic acid and 2% by the alkaline solutions leaving an insoluble residue of

about 60%. The collagenous protein from the acid extracts was precipitated by the addition of sodium chloride. Nitrogen equivalent to 0·3% and 0·2% of the total nitrogen of the macerate remained in solution in the citrate and acetic acid extracts respectively. The citrate extracts were then combined, dialysed to remove sodium chloride and reduced in volume. The acetic acid extracts were treated similarly. The volume of the solutions involved was large, nearly 50 litres. Dialysis was carried out in seamless cellulose tubes 0·5 to 1 inch in diameter and 3 to 4 feet in length. These were mounted in glass tubes of 2½ inch diameter through which water was circulated. For concentrating a stream of air was blown through the tubes in place of water. In this way it was possible to remove upwards of 600 ml. water overnight at ordinary laboratory temperatures, or using four of these tubes in parallel, 2·5 litres. In all, about 200 mg material was obtained from the citrate extracts and 100 mg from the acetic acid extracts. The alkaline extracts were each treated separately. They were neutralised, reduced in volume and precipitated with alcohol. Amounts ranging from about 300 mg in the first extract, to 36 mg in the last, were recovered.

Amino Acid Composition of Protein Fractions.

Hydroxyproline and tyrosine determinations were made on a number of the protein fractions isolated (Table 5). These give some indication of their identity. The approximate collagen content of the various fractions can be calculated from their hydroxyproline contents (col. 5). The value of 8·22 for hydroxyproline-nitrogen as a percentage of collagen total-nitrogen found for the purified citrate-soluble collagen was used in making these calculations. The precipitates obtained from the citrate and acetic acid extracts are mainly collagen, but some non-collagenous protein appears to be carried down with them. The protein remaining in solution is obviously not collagen. The very

TABLE V.

HYDROXYPROLINE AND TYROSINE CONTENT OF EXTRACTED FRACTIONS

	Hydroxy-proline N as % Total-Nitrogen	Tyrosine	% Collagen
Citrate Extracts			
Protein precipitated, Extract 2 ...	7·95	0·19	97·0
„ 4 ...	7·88	0·29	95·8
„ 7 ...	8·15	0·26	99·3
Not precipitated	0·1	0·56	1·2
Acetic Acid Extracts			
Protein precipitated, Extract 2 ...	7·47	0·30	90·6
„ 4 ...	7·73	0·28	94·2
„ 7 ...	7·66	0·29	93·3
Not precipitated	0·19	1·67	2·3
Alkaline Extracts			
Protein precipitated, Extract 5 ...	0·42	2·33	5·1
„ 7 ...	0·93	0·91	11·3
„ 9 ...	1·58	1·54	19·3
Calf skin after Extraction	7·62	0·31	92·9

low hydroxyproline content indicates the presence of a very small amount of collagen, but considering the relative proportions of the two proteins present, the precipitation of the collagenous fraction has been remarkably complete. The protein fractions isolated from the alkaline extracts also appears to be non-collagenous, but the collagen content tends to increase as extraction proceeds.

Complete amino acid analyses of the citrate-soluble non-collagenous fraction and the alkali-extracted fraction have been carried out. These are shown in the form of nomograms in Fig 8 and compared with that of the purified citrate-soluble collagen.

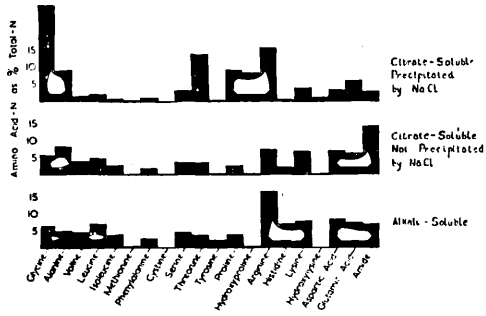

FIG. 8.
Comparison of amino acid composition of protein fractions extracted with citrate buffer and dilute alkali.

In addition to their low hydroxyproline and high tyrosine contents, the amino acid composition of the non-precipitated material from the citrate extract differs considerably from that typical of collagenous proteins, the glycine, proline and arginine contents are lower, and the leucine, isoleucine, valine, lysine, histidine, aspartic and glutamic acids and amide-nitrogen contents are higher.

The alkali-soluble fraction is similar in composition but there are certain differences from the citrate-soluble non-collagenous fraction which cannot be accounted for by its greater collagen content, 5% in this particular sample. The arginine, histidine, leucine, isoleucine, proline and dicarboxylic contents are higher than in the citrate soluble fraction, and the amide content is lower.

The formation of humin on hydrolysis of the citrate-soluble non-collagenous protein and the alkali soluble fraction suggests the presence of sugars but these have not yet been investigated.

Discussion

It would appear from these results that apart from the interfibrillary proteins and elastin, the remaining collagenous tissue of the skin is composed of a number of different protein fractions. First, the collagenous fractions which are characterised by a high hydroxyproline but which apparently differ in solubility ; there is the fraction which is soluble in dilute salt solutions and in phosphate buffer at pH 9·0, the fraction soluble in citrate buffer and finally the remaining insoluble collagenous fraction. These probably all consist

basically of collagen proper but differ in the amount of non-collagenous constituents associated with them.

Reticulin also falls into this group, examination with the electron microscope[36-38] indicates that it consists of small collagen fibres lying in all directions in one plane and embedded in an amorphous matrix. A recent paper by Windrum, Kent and Eastoe[39] indicates that the protein fraction must be almost entirely collagen, but that it is associated with 4% of carbohydrate and 10–12% of bound lipid.

Gross, Highberger and Schmitt[11] consider that the salt-soluble collagen, which appears to be the true precursor of the insoluble collagen fibres, is present in solution in the interfibrillary fluids, while that soluble in citrate-buffer is actually dissolved from the freshly formed fibres.

There appear to be one or possibly two non-collagenous protein fractions. The question of whether the citrate-soluble fraction consists of plasma proteins not completely removed by the phosphate arises. The way in which the amounts of non-collagenous protein nitrogen decrease in successive extracts does not suggest that this is so, and its composition, though similar, shows certain differences from that typical of albumins and globulins; for instance, the aspartic acid content is higher than the glutamic acid content.

The fraction extracted with alkali appears different again. The hydroxyproline content suggests it is more contaminated with a collagenous protein but this cannot account for the differences.

It seems likely that non-collagenous fractions are associated with carbohydrate for there was appreciable humin formation on hydrolysis.

It is possible that these fractions arise from protein-polysaccharide complexes present in the ground substance. Their removal seems to run parallel with reduced stability of the collagenous tissue, e.g. prolonged treatment is known to facilitate the conversion of collagen to gelatin. Also the ready conversion of the citrate-soluble collagen to gelatin suggests that separation from the non-collagenous fraction reduces stability.

It is interesting to see how far the effect of this information regarding the soluble constituents of skin influences present views on the pretanning processes.

The question of the extent to which these soluble fractions should be removed is obviously important. It is fairly generally agreed that the interfibrillary protein should be at least partially removed. This takes place to some extent during soaking, especially if the soak contains sodium chloride or is slightly alkaline. Their removal is continued during liming and possibly during bating where the enzymes will act more readily on the soluble constituents of the skin. If the skin is dried or stored in the salted condition for long periods, the solubility of these interfibrillary albumins and globulins will be decreased and hence their removal will be more difficult.

How far the more soluble collagenous constituents and the non-collagenous protein fractions should be removed is more difficult to say and, of course, depends on the type of leather under consideration. As stated earlier, removal of the non-collagenous fractions appears to lead to decreased

stability. In its earlier stages this is equivalent to opening up and it is, therefore, almost certainly desirable to remove some of this material in order to get proper penetration of tan. If a very flexible leather is wanted, as for example gloving leather, then it is desirable to remove more than if a firm upper leather is required. Evidence suggests that the non-collagenous protein is removed relatively quickly from macerated skins in alkaline solutions, but, how far this will be true when dealing with a whole skin in which diffusion of the protein will be much slower remains to be investigated. Although the alkali will bring it into solution it may not necessarily remove it from the skin. Here bating may play a part in further degrading it and so facilitating its removal.

The desirability of removing the acid-soluble collagen is not certain. The presence of soluble proteins in the skin in general seems to lead to sticking together of the fibres on drying and hence some removal may be advisable, especially with the skins of young animals which contain a relatively high proportion. It would be interesting to know what effect soaking in dilute acid solution would have on such skins, in particular calf. The use of the old bran drenches containing organic acids may have had some significance in this respect.

Acid-soluble collagen is reported to be more readily attacked by enzymes than collagen[6]; some degradation may, therefore, occur during storage. It is possible that the benefits attributed to keeping calfskins in the salted condition may be partially derived from the breakdown of this protein, and hence its more ready removal at later stages. Breakdown may also occur during bating, leading to further removal at this stage.

Although the amounts of the more soluble constituents which are now known to be present in skin are small, they have already been shown to play an important part in the formation, and regeneration of collagen and in many of the diseases of the collagen system. It is probable that they also have an important influence on the behaviour of the skin during processing. In any event, it must be agreed that the skin is a very complicated system with which to deal, and that in considering the pretanning processes, their effect on a variety of constituents must be taken into account.

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THE COMPOSITION OF EPIDERMIS.

By J. H. Bowes and R. G. Elliott.

SUMMARY.

Epidermis has been separated from ox hide by means of treatment in warm water, and the amino acid composition has been determined. The majority of amino acid analyses previously reported for epidermis have been made on material obtained from abnormal sources, such as that from cornified areas and from subjects suffering from exfoliative dermatitis, and this is the first complete analysis of normal material which has been made. Epidermis is generally classified as a "pseudokeratin," and it differs markedly from the normal keratins, such as hair, in its much lower cystine content. It is now shown that its amino acid composition differs also in a number of other respects. The relative swelling and solubility of epidermis, hair, and wool in alkaline and sulphide solutions have been examined.

The epidermis consists of layers of cells, those nearest the surface of the dermis proper being alive and multiplying, and those further away gradually becoming more keratinised and dying. It forms a continuous layer covering the surface of the skin and the walls of the hair follicles which dip down into the grain layer of the skin. Solubilisation of the less keratinised layers at the base of the hair follicles may be a contributory factor in the loosening of hair, and the removal of the epidermal layer is a necessary feature of the pre-tanning processes. Hence, some knowledge of its composition and its properties is desirable for the further understanding of the unhairing process.

Although a few analyses of epidermis have been made in the past, they are incomplete, being mainly confined to the determination of cystine and methionine. Also, owing to the difficulties of separating normal epidermis from the dermis proper, the majority of these analyses have been carried out on abnormal material such as that obtained from cornified areas or from persons suffering from exfoliative dermatitis. The possibilities of removing the epidermis by such mild treatments as soaking in sodium chloride solutions[1,2,3], or warming in water to 60°[2,3,4], have now made it relatively easy to obtain appreciable amounts of normal epidermis in a reasonably pure condition. Buechler and Lollar[2,5] have carried out cystine and methionine determinations on such material ; their methods for determining cystine also include cysteine, and from the results of two methods of determination, they conclude that both the normal and homo-derivatives of cystine and cysteine are present. They were only able to account for 75% of the total sulphur as cystine derivatives and methionine.

From these and other determinations it is clear that epidermis has a much lower cystine and cysteine content than keratins, and it has generally been classified as a pseudokeratin[6]. Evidence suggests that the proportion of sulphydril groups decreases towards the outer, more cornified layers of the epidermis[7, 8]. Working on the thick epidermis which can be obtained from the nose of the cow, Rudall[7] has shown that the thermal stability of the epidermis also varies at different levels, the inner layer (or *stratum mucosum*) shrinking at about 65°, and the outer layer (or *stratum corneum*) at 80°. This shrinkage of the inner layers at 65° is no doubt related to the loosening of the epidermis

from the dermis which occurs on treatment in water at this temperature. Rudall was also able, by dispersion in urea, to separate the epidermis into two major components, one fibrous (with a low sulphur content) and one non-fibrous (with a much higher sulphur content). The relative amounts of the two components varied throughout the thickness, the outer layers consisting predominantly of the fibrous protein. Thus, epidermis cannot be considered as homogeneous, and variations in the proportion of the two components identified by Rudall may account for the differences in sulphur and cystine contents reported for epidermis from different sources.

In the present investigation, epidermis has been separated from ox hide by treatment in warm water at 60°, and a complete amino acid analysis carried out. The relative solubility of epidermis, wool, and hair in sodium hydroxide and sodium sulphide solutions has also been determined.

Experimental Technique.

(i) PREPARATION OF EPIDERMIS.

Hide pieces were obtained immediately after flaying and cut into strips 1 in. × 3 in. These strips were immersed in warm water at 60° for 5 min., the epidermis was then peeled off in small pieces, leaving the greater part of the hair in the skin. The hair remaining in the epidermis was removed with tweezers, using a lens to ensure that the removal was complete. The removal of these hairs left small holes where the surrounding epidermis had been torn away with the hair. The epidermis obtained was stored under acetone, degreased with light petroleum in a soxhlet, and air dried.

(ii) METHODS.

Ash[9], moisture[9], total-N[9], amide-N[9], and total-S[10] were determined as previously described. Amino acids were determined by the technique of Moore and Stein[11] (see Bowes, Elliott and Moss[12]) following hydrolysis with 6N HCl under reflux for 24 hr.

Cystine plus cysteine was determined by oxidation with performic acid, followed by separation of the cysteic acid formed on Dowex 2 and its colorimetric determination with ninhydrin[13]. This method does not distinguish between cystine and cysteine, but allows of the partial separation of cysteic and homocysteic acids arising from cystine and homocystine, respectively.

Tryptophan was determined by a modification of the method of Spies and Chambers[14,15], both directly and after preliminary hydrolysis in N NaOH under reflux for 2 hr. 100 mg. epidermis was treated with 100 ml. 19N H_2SO_4 containing 0.3 g *p*-dimethylamino-benzaldehyde and left in the dark. A standard solution of tryptophan in the same reagent was treated similarly. After 24 hr., 5 and 10 ml. aliquots of the unknown and suitable aliquots of the standard containing between 1 and 8 mg. of tryptophan were taken, made up to 10 ml. with 19N H_2SO_4 containing the *p*-dimethylaminobenzaldehyde reagent, 0.1 ml. of .05% sodium nitrite added to each and left in the dark for 0.5 hr. The blue colour developed was then read at 590 mμ, using a a Uvispek spectrophotometer. All the epidermis had not dissolved in the 24 hr., and after a

further 72 hr. similar aliquots of unknown and standard were treated as before. A slightly higher result was obtained. A similar procedure carried out directly on an aliquot of the alkaline hydrolysate of epidermis, and compared with tryptophan standards similarly treated, gave an appreciably lower result. This was probably due to destruction of tryptophan; it is reported that this occurs in the presence of cystine[16].

(iii) Water Uptake and Solution in Sodium Hydroxide and Sulphide Solutions.

1 g. of protein was placed in 100 ml. of sodium hydroxide solution pH 12.6, or in 0.1 % sodium sulphide solution pH 12.38, in a stoppered flask for 3 days at 20°C. The protein was then filtered on a weighed sintered-glass crucible with gentle suction. The crucible and protein were weighed at once, and again after drying at 105° for 18 hr. From the weight of the dried protein and the weight of water lost during drying, the percentage water uptake was calculated. An approximate correction was made for the amount of water held in the interstices of the glass disc in the first weighing of the wet protein and crucible. This correction was 0.9 to 1.1 g, and since the total weight of water held was generally between 4 and 12 g, the error due to inaccuracies in estimating the correction was small. The nitrogen in solution was determined by the usual microkjeldahl procedure[9], and the protein dissolved calculated, using values of 16.60 (this paper), 16.50[17], and 15.6[18], respectively, for the nitrogen content of epidermis, wool, and hair.

Experimental Results.

(i) Separation of Epidermis.

No attempt was made to determine the yield of epidermis per lb. of hide, but it was almost certainly better than that reported by Beuchler and Lollar[2], amounts of the order of 0.7 g. of hair and grease-free material being obtained from 1 sq. ft. or about 2 lb. hide. The epidermis was obtained in the form of pieces about 1 cm^2 or less, with many small holes, corresponding to the tearing away of material adjacent to the mouth of hair follicles.

(ii) Amino Acid Analysis.

The values obtained for the total–N and total–S are almost identical with those reported by Beuchler and Lollar[2,5] for similar material. Rather lower values for nitrogen have been quoted by other workers for cornified epithelium, together with appreciably higher values for total-sulphur (see Table I).

The amino acid composition of epidermis expressed in various ways in given in Table I and is compared with determinations made by other workers at various times.

Only 89 % of the total–N has been accounted for. No other unidentified peaks were observed in the Moore and Stein chromatographic procedure and this low recovery may be due to greater destruction of serine and threonine, than takes place with other proteins, low recovery of tryptophan, or over-all losses of amino acids. It may be noted that the recent analysis of hair keratin

TABLE I.

AMINO ACID COMPOSITION OF EPIDERMIS.

	Amino Acid–N as % Total–N	g. per 100 g.	g. residues per 100 g.	m. mol amino acid per g.	Values from literature, g. per 100 g.
Total—N		16·60			
Total—S		0·77			11·3(*a*), 15·1(*c*), 15·2(*g*), 16·9(*h*), 17·5(*i*) 0·70(*a*), 0·50(*b*), 1·09(*f*), 0·79(*h*), 0.85(*i*), 1·13(*k*)
Glycine	11·64	10·36	7·87	1·38	
Alanine	4·09	4·32	3·44	0·484	
Valine	2·40	3·34	2·82	0·285	
Leucine	5·36	8·33	7·19	0·635	
Isoleucine	2·13	3·31	2·86	0·259	
Phenylalanine	2·04	3·99	3·55	0·241	2·9(*e*)
Tyrosine	2·19	4·70	4·23	0·259	5·70(*c*), 3·42(*d*), 5·0(*e*)
Tryptophan	0·78	0·95	0·86	0·046	1·49(*c*), 1·80(*d*), 0·05(*e*)
Serine	9·28	11·51	9·57	1·10	17·0(*e*)
Threonine	3·06	4·33	3·67	0·363	3·5(*e*)
Cystine (—S—S— +SH)	1·16	1·66	1·40	0·069	0·17(*h*), 0·55(*i*) } 1·8–2·3(*a*), 2·3(*c*), 3·82(*d*)
Homocystine	—				0·76(*i*) } 3·5(*e*), 1·4(*j*), 2·38(*f*)
Methionine	0·93	1·64	1·44	0·110	1·3(*h*), 1·53(*i*), 1·0(*e*), 2·47(*f*)
Proline	2·00	2·73	2·31	0·237	3·3(*e*)
Hydroxyproline	0·00	—	—	—	
Arginine	10·95	5·65	5·07	3·24	5·91(*d*), 10·01(*c*), 7·9(*e*)
Histidine	2·64	1·62	1·43	0·104	0·64(*d*), 0·59(*c*), 1·9(*e*)
Lysine	6·03	5·22	4·58	0·357	4·68(*d*), 3·08(*c*), 7·1(*e*)
Unknown (2N)	0·16	—	—	0·096	
Aspartic Acid	6·02	9·49	8·21	0·713	8·4(*e*)
Glutamic Acid	8·78	15·30	13·43	1·04	15·9(*e*)
Amide—N	7·28	1·21		0·864	
Hexosamine	0·12	0·25	—	0·104	
Total	89·04		83·93		
Methionine + Cystine — S		0·80			

TABLE II.

COMPARISON OF THE COMPOSITION OF EPIDERMIS WITH VARIOUS TYPES OF KERATIN. g per 100 g. dry protein.

Component	Epidermis (this paper)	Wool (sheep)	Hair (human)	Horn (cattle)	Feather (chicken)
		taken from Ward and Lundgren[17]			
Total—N	16·60	16·2–16·9	15·5–16·9	14·8–16·9	15·0–16·2
Total—S	0·77	3·0– 4·0	5·0– 5·2	3·8– 3·9	2·9
Glycine	10·36	5·2– 6·5	4·1– 4·2	9·6	7·2
Alanine	4·32	3·4– 4·4	2·8	2·5	5·4
Valine...	3·34	5·0– 5·9	5·5–(5·9)	5·3– 5·5	8·3– 8·8
Leucine	8·33	7·6– 8·1	6·4–(8·3)	7·6– 8·3	7·4– 8·0
Isoleucine	3·31	3·1– 4·5	(4·7) – 4·8	4·3– 4·8	5·3– 6·0
Phenylalanine ...	3·99	3·4– 4·0	2·4– 3·6	3·2– 4·0	4·7– 5·3
Tyrosine	4·70	4·0– 6·4	2·2– 3·0	3·7– 5·6	2·0– 2·2
Tryptophan	0·95	1·8– 2·1	0·4– 1·3	0·7– 1·4	0·7
Serine	11·51	7·2– 9·5	7·4–10·6	6·1	4·4– 4·8
Threonine	4·33	6·6– 6·7	7·0– 8·5	6·1	4·4– 4·8
Cystine	1·54	11·0–13·7	16·6–18·0	10·5–15·7	6·8– 8·2
Methionine	1·64	0·5– 0·7	0·7– 1·0	0·5– 2·2	0·4– 0·5
Proline	2·73	5·3– 8·1	4·3–(9·6)	8·2	8·8–10·0
Hydroxyproline ...	not found	—	—	—	—
Arginine	5·65	9·2–10·6	8·9–10·8	6·8–10·7	6·5– 7·5
Histidine	1·62	0·7– 1·1	0·6– 1·2	0·6– 1·1	0·3– 0·7
Hydroxylysine ...	not found	—	—	—	—
Lysine	5·22	2·8– 3·3	1·9– 3·1	2·4– 3·6	1·0– 1·7
Aspartic Acid ...	9·49	6·4– 7·3	3·9– 7·7	7·7– 7·9	5·8– 7·5
Glutamic Acid ...	15·30	13·1–16·0	13·6–14·2	13·8	9·0– 9·7
Amide	0·92	1·10–1·37	1·17	1·14	1·09

by Lucas and Long[18], using microbiological methods, also accounted for only 93% of the total-N as amino acids and amide.

All the sulphur is accounted for as cystine or methionine. This is contrary to the findings of Buechler and Lollar[2,5] who were only able to account for about 76%. The present figures for both cystine derivatives and methionine are, however, rather higher than the figures reported by these authors. The shape of the cysteic acid peak obtained in the chromatographic procedure does not indicate the presence of homocystine in addition to cystine as was suggested by Buechler and Lollar[5].

Footnotes to Table I.

(*a*) Wilson and Lucas, *J. biol. Chem.*, 1927, **73**, 543. Human skin.
(*b*) Giroud, Bulliard, and Giberton, *C.R. Soc. biol., Paris*, 1929, **100**, 1024. Horse burr.
(*c*) Wilkerson, *J. biol. Chem.*, 1934, **107**, 377, Stratum corneum of foot from human with exfoliative dermatitis.
(*d*) Echstein, *Proc. Soc. exp. Biol. & Med.*, 1934/5, **32**, 1573. From human.
(*e*) Block, *J. Cosmetic Chem.*, 1951, **2**, 235. Cornified epithelium from foot.
(*f*) Wilkerson and Tulane, *J. biol. Chem.*, 1939, **129**, 477—as (*c*).
(*g*) Eichelberger and Roma, *J. invest. Derm.*, 1949, **12**, 125. Epidermis of dog, removed by warm water.
(*h*) Buechler and Lollar, *J.A.L.C.A.*, 1949, **44**, 359. Bovine epidermis separated by warm water.
(*i*) Buechler and Lollar, *J.A.L.C.A.*, 1950, **45**, 503. As (*h*) .
(*j*) Van Scott and Flesch, *Science*, 1954, **119**, 70. Layers of plantar skin.
(*k*) Rudall, " Advances in Protein Chemistry," 1952, **7**, 253. Cow nose corneum.

TABLE III.

WATER UPTAKE AND SOLUTION OF EPIDERMIS, HAIR AND WOOL IN SODIUM HYDROXIDE AND SULPHIDE SOLUTIONS.

	Sodium Hydroxide			Sodium Sulphide		
	Final pH solutions	g Water per 100 g. protein	Protein dissolved, %	Final pH of solutions	g Water per 100 g. protein	Protein dissolved, %
Epidermis ...	12·21	1798	23·5	12·07	2876	43·0
Wool	12·28	339	1·6	12·14	708	36·4
Hair	12·31	163	0·68	12·22	503	9·6

Values obtained by other workers for certain of the amino acids are given in the last column of Table I. The epidermis used was from a variety of sources, in many cases from cornified areas, such as the foot or from cases of exfoliative dermatitis, and the values are not, therefore, necessarily representative of normal epidermis. The values for cystine and methionine, which are the greatest in number, vary considerably and are almost certainly influenced by the source of the epidermis and its method of separation, e.g., the highest values of 2.3% for cystine and 2.4% for methionine were obtained on cornified epithelium.

(iii) WATER UPTAKE AND SOLUBILITY IN ALKALINE SOLUTIONS.

The relative water uptake and solution of epidermis, wool, and hair in sodium hydroxide and sulphide solutions are given in Table III. The swelling and solubility of epidermis in sodium hydroxide solutions is very much greater than that of either wool or hair: this is probably due to its much lower cystine content. Hair, with the highest cystine content, swells less and is less soluble than wool. The water uptake and solubility of all three proteins are much greater in sodium sulphide than in sodium hydroxide solutions at the same pH value. This increase is presumably due to breakdown of the disulphide link, and is least marked with epidermis in which the cystine content is relatively low.

Discussion.

Epidermis cannot be regarded as a homogeneous protein, as it is well known histologically that its structure changes throughout its thickness, the inner layers consisting of living cells and the outer layers of more cornified material. The proportion of —S—S— compared with SH groups also increases with increasing distance from the dermis[7,19].

However, it is of general interest to have an over-all amino acid analysis of the epidermis as it separates from the skin in normal animals, not only for comparison with that of hair, wool, and other keratins, but also as a basis for comparison with similar material obtained from animals with skin troubles involving the epidermis. A study of the composition of epidermis from different species would also be of interest.

The composition of epidermis obviously differs appreciably from that of

typical keratins such as wool, hair, and horn (see Table II); not only is the cystine (cystine and cysteine) very much lower, but there are appreciable differences in certain of the other amino acids; the methionine and glycine content is higher than that of the majority of keratins, and in this respect epidermis most nearly resembles horn. The lysine, aspartic acid, and histidine contents are higher than that of the hard keratins; while the proline, threonine, and to a lesser extent the valine and arginine, values are lower. The most important difference between epidermis and keratins such as wool and hair is its much lower cystine and cysteine content. Since a large proportion of thiol groups are considered to be present, this means that there can be relatively few disulphide bonds. This presumably accounts for the much greater swelling and solubility of epidermis compared with wool and hair, while its less organised structure makes it more soluble than collagen. For these reasons, epidermis will be preferentially dissolved in the pre-tanning processes; solution of the epidermal layer lining the hair follicle is probably one of the factors involved in the loosening of the hair and wool. It is probably mainly by this mechanism that wool or hair is loosened by the action of alkalis alone, such as sodium hydroxide and ammonia. In the presence of sulphide, breakdown of disulphide bonds occurs and the swelling and solubility of wool and hair is increased to a greater extent than that of epidermis. Under these circumstances, the hair shaft is attacked, and loosening of the hair is then due to degradation of the hair root and shaft. After the skin has been salted or dried, the inner layers of the epidermis are no longer sufficiently soluble for the hair to be loosened satisfactorily by alkalis alone, and sulphide is necessaryy to attack the disulphide bonds of the hair or wool root.

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FORMALDEHYDE TANNING.

Part I.—The Combination of Formaldehyde with Collagen, Keratin (Hair), and Silk Fibroin.

By
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(Reprinted from the *J.I.S.L.T.C.*, 1939, **23**, pp. 365 to 385).

Introduction.

Many different types of raw material destined for various purposes are treated with formaldehyde either alone or in conjunction with some other tanning material.

Formaldehyde tanning produces a white leather similar in appearance to that of an alum tanned skin. It has one great advantage, however, over this tannage, in that it is more resistant to the action of water and for this reason it is generally used when a white washable leather is required, as for gloves. For soft washable leathers it is becoming increasingly common first to tan the skin with formaldehyde and then to oil dress. White military leathers may be tanned either with formaldehyde or alum, but since these are liable to have to withstand the action of moisture, alum tanning is not so satisfactory as formaldehyde tanning.

Formaldehyde is also used for tanning fur skins. Formaldehyde tanning is usually followed by an oil dressing in order to increase the strength and ' run ' of the fur skins. Sheepskins in the wool are also sometimes tanned with formaldehyde but it is very difficult to treat the skins so that the grain and epidermis are sufficiently soft and pliable when tanned in this way.

Formaldehyde has occasionally been used as a pre-tanning agent for heavy vegetable tanned leathers. The object of this pre-treatment is to increase the rate of penetration of the vegetable tan and according to Houben (1934) may be given so as to fix the limed pelt in the plumped condition or alternatively may be given to the pelt after deliming. Previous treatment with formaldehyde has an effect similar to deamination and causes vegetable tannins and chromium to be taken up less readily. Gerngross and Roser (1922) point out this inhibitory effect and advise caution in the use of formaldehyde on this account. It is this effect, however, which is the basis of the pre-treatment, since it gives a method of lessening the astringency of tan liquors on the skin, enabling the treated pelt to be placed in more concentrated liquors, thus shortening the tanning process.

Formaldehyde tanning is usually an empirical process carried out by following some recipe, one of the most common being the Pullman-Payne process (1898). Little appears to be known of the many factors which may influence the tanning process and thus alter the character of the finished product. A number of papers have appeared in the literature, however, dealing with the reaction between formaldehyde and amino acids and proteins.

Section I.—Chemistry of the Tanning Action of Formaldehyde.

Protein molecules consist of amino acids united together by means of their carboxyl and α amino groups to form a backbone of peptide groups with side chains protruding. The side chains may contain polar groups which vary in character with the different proteins. Free amino groups are present in some of the side chains in all proteins, but although the number varies with the protein it is never large. The free amino groups are mainly derived from the residues of the diamino acids, lysine and arginine, and are also present as terminal groups of the backbone.

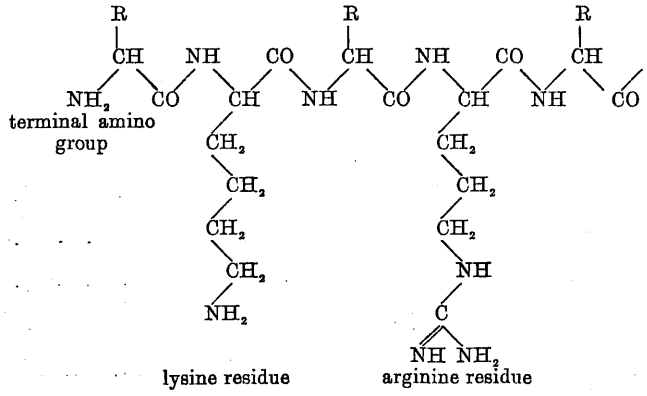
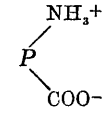


Fig. 1

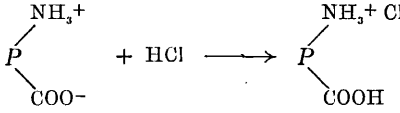
The polypeptide backbone and side chains of lysine and arginine residues are shown diagramatically in Fig. 1.

It is now fairly generally agreed that proteins may be regarded as forming zwitterions (i.e. ions carrying both positive and negative charges), by the simultaneous ionisation of the basic and acidic groups. Harris (1926) and Harris and Birch (1930) have shown that only the zwitterion theory can account for the titration curves of amino acids, polypeptides and proteins. According to this theory proteins exist under almost all conditions as zwitterions, the degree of ionisation of any one group being determined by the pH value.

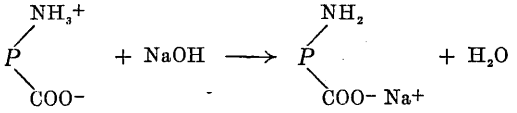
At the iso-electric point the proteins carry equal numbers of positive and negative charges and the simplest case may be represented as:—



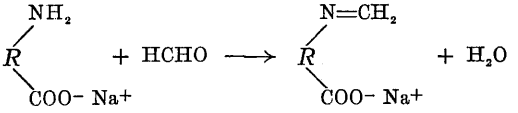
In acid solution proteins form salts with acids, and the ionisation of the carboxyl group is suppressed. For instance, in the case of hydrochloric acid, the protein forms chlorides by electrovalent links at its amino groups.



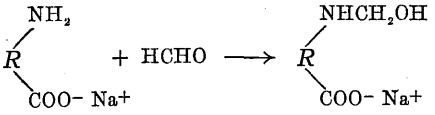
conversely in alkaline solution.



The tanning action of formaldehyde is generally considered to depend on its power of uniting with the amino groups present in collagen, although other groups may also be involved. Experiments both with amino acids and also with proteins by Schiff (1900-1902) and other workers have resulted in the general acceptance of the theory that combination occurs at the amino groups with the formation of methylenimino groups:—



Later workers, however, have suggested a slight modification of this reaction. For instance, Balson and Lawson (1936) have suggested the following mechanism:—

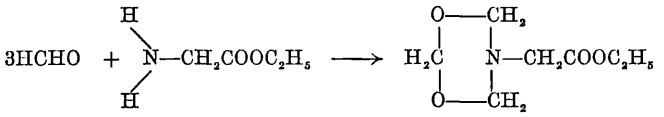


In either case, the basic amino groups are converted into neutral groups, causing an increase in the acid character of the protein, and a consequent

shifting of the isoelectric point towards the acid range. Gerngross and St. Bach (1922 and 1923) report that treatment with formaldehyde shifted the isoelectric point of gelatin from pH 4.75 to 4.3 in one experiment and from pH 5.05 to pH 4.6 in another experiment. In the case of hide powder the isoelectric point was shifted from 4.8-5.6 to 3.8-4.2 (Gustavson, 1929).

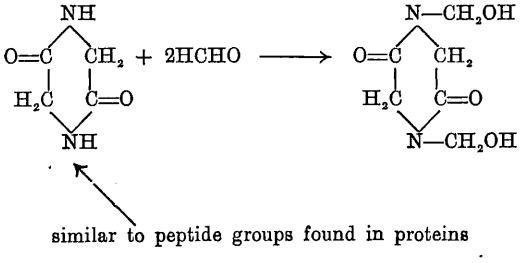
In practice, formaldehyde tanning is always carried out in alkaline conditions. From theoretical considerations chemical combination would be expected to take place more readily at higher pH values since it would be expected that formaldehyde would more readily react with the uncharged than with the charged amino groups. As the pH value is increased, greater proportions of the amino groups in the lysine and arginine residues and also at the ends of the polypeptide chains of the proteins become uncharged and therefore more readily available for reaction with formaldehyde.

An increase in the amount of formaldehyde fixed with an increase in pH value has been reported by a number of workers and has also been observed in this investigation. If, however, formaldehyde only combined with the free amino groups as shown in Fig. 1, comparatively small amounts would be fixed by proteins (see Table II). It is generally agreed that on prolonged treatment collagen is able to fix more formaldehyde than can be accounted for in this way. There are two possibilities, either that more than one molecule of formaldehyde can be fixed by each amino group, or that other groups in the protein molecule are able to combine with formaldehyde under suitable conditions. Considering the first possibility, although Bergmann (1923) has shown that glycine ethyl ester will react with three molecules of formaldehyde in the following manner:—

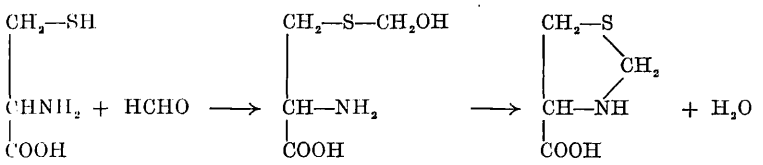


this compound is not very stable and with loss of formaldehyde is converted into the methylenimino compound of Schiff. It seems unlikely, therefore, that each free amino group in a protein would form a stable compound with more than one molecule of formaldehyde.

The second possibility is certainly more probable since evidence of formaldehyde combining with imino or sulphydril groups can be brought forward. Evidence of the possibility of combination at the peptide group can be drawn from the work of Cherbuliez and Feer on diketopiperazine. They suggested the following reaction which was later confirmed by Bergmann (1923-1924).



As evidence of the possibility of the combination of formaldehyde with proteins at the sulphydril group the work of Ratner and Clark (1937) may be quoted. These workers have shown that in the case of cystine derivatives combination with formaldehyde takes place at the sulphydril group more readily than at the amino group. In the case of cysteine this reaction is followed by ring closure.



In the presence of formaldehyde cystine in hair or wool tends to be reduced to cysteine. The sulphydril groups will then probably combine with formaldehyde.

It seems, therefore, probable that the tanning of collagen by formaldehyde, in addition to combination at the amino groups, may involve reaction with imino groups in the side chains or at the peptide groups, while in the case of hair or wool combination probably takes place at the sulphydril groups also.

There is evidence that, although the reaction between proteins and formaldehyde is fairly rapid at first, prolonging the time of reaction causes increased quantities of formaldehyde to be slowly fixed. Stiasny (1936) has suggested that in formaldehyde tanning two reactions are involved, a rapid reaction with amino groups and a slow reaction with the more numerous peptide groups. As has already been discussed the extent to which the amino groups react with formaldehyde will depend on the pH value of the solution, but there are many other factors which are known to influence the total fixation by the protein, in particular, temperature, time and concentration of formaldehyde.

An examination of some of the papers published shows that the amount of formaldehyde fixed by proteins varies widely, chiefly owing to the different experimental conditions employed (see Table I). In the majority of cases the amount of formaldehyde taken up by the protein has been estimated by analysis of the solution before and after use. This method is open to error owing to the quantity of formaldehyde dissolved in the solution in the interstices of the protein and also to the volatility of formaldehyde and the consequent ease with which this may be lost during the time of treatment. This probably accounts for the very high values obtained by Moeller (1921) for the amount of formaldehyde taken up by hide powder. Since he used very high concentrations of formaldehyde over a long period of time, it is probable that quite a considerable amount of formaldehyde was lost from the solution, other than that taken up by the protein. In his case the hide powder was given a short pre-treatment with the formaldehyde before treatment with the concentrated solutions for longer periods. He considers that chemical combination is only involved in the pre-treatment and that the second process is one of adsorption.

TABLE I

Name	Date	Protein	Concentration of formaldehyde %	pH	Time	Temperature	g. Formaldehyde taken up per 100 g. protein	Method of estimation	Ratio of liquor to goods
Benedicenti	1897	Gelatin	0·6	—	—	—	1·35	——	—
Lumière and Seyewetz	1906	Gelatin	10	—	12 hours	—	4·0–4·80	——	—
Möeller	1921	Hide Powder	15 30	(neutral) ,,	1 month ,,	— —	73·64 77·73	Analysis of solution before and after use	22 : 1 ,,
Brotmann	1921	Gelatin 10% gel 7·5% gel 5·0% gel	2 ,, ,, ,,	— — — —	1 week ,, ,, ,,	— — — —	0·98 1·12 1·82 2·12	Analysis of solution before and after use	— — — —
Thomas, Kelly and Foster	1926	Gelatin	5 ,, ,, ,, ,, ,, 10 ,, ,, ,, ,, ,, ,,	4 6 8 9 10 11 4 6 8 9 10 11	24 hours ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,,	4°C. ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,,	0·76 0·79 0·97 1·34 1·48 2·11 1·22 1·26 1·42 1·77 2·61 3·99	Analysis of solution before and after use	40 : 1 ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,,

TABLE I—continued

Name	Date	Protein	Concentration of formaldehyde %	pH	Time	Temperature	g. Formaldehyde taken up per 100 g. protein	Method of estimation	Ratio of liquor to goods
Thomas, Kelly and Foster	1926	Gelatin	10	4	1 week	Room Temperature	0·04		80 : 1
			,,	6	,,	,,	0·68	Direct estimation of formaldehyde in tanned protein after drying at 37°	,,
			,,	8	,,	,,	0·71		,,
			,,	9	,,	,,	0·69		,,
			,,	10	,,	,,	0·28		,,
Thomas, Kelly and Foster	1926	Gelatin	0·05	7·0	24 hours	Room Temperature	0·04	Analysis of solution before and after use	20 : 1
			1·0	,,	,,	,,	0·22		,,
			2·0	,,	,,	,,	0·34		,,
			3·0	,,	,,	,,	0·37		,,
			5·0	,,	,,	,,	0·56		,,
			10·0	,,	,,	,,	0·88		,,
			20·0	,,	,,	,,	1·82		,,
			0·44	9·3	,,	,,	0·08		,,
			0·88	,,	,,	,,	0·10		,,
			1·75	,,	,,	,,	0·16		,,
			2·63	,,	,,	,,	0·18		,,
			4·38	,,	,,	,,	0·29		,,
			8·76	,,	,,	,,	0·56		,,
			17·52	,,	,,	,,	1·12		,,
Thomas, Kelly and Foster	1926	Hide Powder	20	9	24 hours	Room Temperature	13·5	Analysed for hide substance and difference from 100% taken as formaldehyde	50 : 1
Anderson	1934	Hide Powder	0·12	2·6	3 hours	—	0·1	Solution filtered and formaldehyde in filtrate estimated by Romijn's method	25 : 1
			,,	3·8	,,	—	6·05		,,
			,,	4·9	,,	—	0·5		,,
			,,	6·5	,,	—	1·4		,,
			,,	7·1	,,	—	2·8		,,

Thomas, Kelly and Foster (1926) in a paper presenting their results give a comprehensive review of the literature published on the subject prior to this date. These authors have measured the amount of formaldehyde fixed by gelatin and hide powder by estimation of the loss of formaldehyde from the solution and by analysis of the " tanned " material. The difference between the protein as estimated by Kjeldahl nitrogen and the weight of tanned protein was taken as formaldehyde. They find that the amount fixed increased with the alkalinity and also with the concentration of the formaldehyde solution employed. The greatest fixation was obtained between pH 6 and 9 and the presence of high concentrations of salt increased the rate at which this occurred. The results were rather erratic with hide powder owing to hydrolysis taking place, but the tanning effect was greatest between pH 7 and 9.

Gerngross and Gorges (1926) measured the degree of tannage by the resistance of the tanned hide powder to hydrolysis in hot water. They found a 25 per cent. increase in resistance to hydrolysis between pH 4 and 7, followed by a further sharp increase to a maximum value at pH 8.7. Above pH 9 there was a marked hydrolysis of the hide powder.

More recently Theis and Schaffer (1936) have made measurements of the shrinkage temperature of formaldehyde leather tanned under various experimental conditions and have employed this method as a means of assessing the degree of tannage. The raw material was pickled calf skin, depickled and adjusted to the required pH before tanning. Their results showed that tanning action, as indicated by rise in the shrinkage temperature, increased with time, concentration of formaldehyde, temperature and pH value of the solution. No further increase in shrinkage temperature was observed after 6 hours when the concentration of formaldehyde was 1.5 per cent. It is possible that this apparent equilibrium indicates the completion of the reaction with the amino groups. Above 2 per cent. concentration of formaldehyde there was little further increase in the shrinkage temperature. There was little or no tanning action below pH 5.0, but an increase to pH 7.0 caused a marked increase in the shrinkage temperature. These workers also studied the effect of the presence of sodium acetate, formate, sulphate, thiosulphate and chloride in the tanning solution. Comparing the initial pH values of the solutions and the increase of shrinkage temperature, they concluded that all, with the exception of the chloride, increase the shrinkage temperature, the acetate and the formate being the most effective. It will be observed, however, that if the final pH values are plotted against the increase in shrinkage temperature, the presence of these salts slightly decreases the shrinkage temperature. The apparent increase inferred by Theis and Schaffer was probably due to the buffer action of these salts. The presence of sodium chloride and sulphate, especially the latter, was found to reduce the tendency to give a cracky grain.

Casaburi and Cantarella (1937) have extended this work to the tanning of pickled lambskins and have arrived at similar conclusions. The shrinkage temperature was again taken as a measure of the degree of tanning and the

experimental conditions were carefully controlled. Equilibrium was reached after 1½ hours, whereas Theis and Schaffer found that equilibrium was not reached for 6 hours. The concentration of formaldehyde used was, however, slightly higher, viz., 2 per cent., as compared with 1.5 per cent. used by Theis and Schaffer, but it is probable that the more rapid attainment of equilibrium was due to the difference in the nature of the calf and lamb pelts, rather than to the difference in the concentration of formaldehyde. Casaburi and Cantarella also studied the effect of the presence of sodium citrate in the tanning solution and found that the use of concentrations up to 2 per cent. caused an increase in the shrinkage temperature.

Other workers have also studied the effect of salts on the tanning action of formaldehyde. Gerngross and Gorges (1926) suggest that excessive swelling of the hide fibres in alkaline solution is harmful and that the presence of salt is necessary to reduce the swelling.

In order to have a better understanding of the process of formaldehyde tanning a comprehensive study of the various factors which influence the reaction has been undertaken. The present paper deals with the influence of the pH value of the solution. Chemical estimations of the amount of formaldehyde fixed by collagen, hair or silk at different pH values have been made, all other influencing factors being kept as nearly constant as possible. Collagen and keratin were chosen as being the two chief proteins occurring in skin, and silk fibroin was chosen as an example of a protein having a simple and compact structure with comparatively few free amino and carboxyl groups.

One of the main difficulties of formaldehyde tanning as it is now carried out, is the prevention of the occurrence of cracky grain when skins are tanned in the wool or fur. Hair is representative of the protein occurring in the epidermal layer, and it was hoped by comparison of the fixation of formaldehyde by hair and collagen under the same conditions, to discover the cause of this cracky grain. Formaldehyde tanning of flesh splits is a comparatively simple process, but it is very difficut to tan satisfactorily full grain skins, even when the hair and epidermis have been removed during liming. Thus there are two special problems, that of tanning the grain in a skin which has been unhaired and also the tanning of the grain and epidermis in a skin which is to be finished in the wool or fur.

Section II.—Influence of the pH value on the Combination of Formaldehyde with Collagen, Keratin (Hair) and Silk Fibroin.

(i) Experimental Methods.

Collagen, which had been purified and acetone-dehydrated (for details see Pleass, 1929), was selected for this work since a uniform stock of raw material, which would be sufficient in amount for the whole investigation, was required. Before treatment the dehydrated collagen was cut into strips about 0.5 cms. in width and 4 cms. in length. The goat hair was kindly supplied by Messrs. J. Salomon & Co. The hair was clipped from the skins, washed thoroughly in running tap water and rinsed in distilled water. When clean, the hair was degreased and dehydrated in acetone, followed by light

petroleum and then air dried. Natural unspun silk after degumming was kindly supplied by the Cotton Research Association.

In each case 5 g. air dry protein was weighed into a stoppered bottle and 200 c.c. of the experimental solutions was added. Solutions covering a wide range of pH values (1-11) were used. The initial concentration of formaldehyde in all these solutions was the same, namely, 0.8 g. formaldehyde per 100 c.c. Tanning was carried out at room temperature for 48 hours. The pH values of the solutions before and after use were determined colorimetrically and whenever practicable (i.e. except at high alkalinities), also by the glass electrode method.

The following method, based on Romijn's method, was used for the estimation of the formaldehyde which had been taken up by the protein. The protein was removed from the experimental solution and thoroughly washed on a filter pump with a solution at the same pH as that of the experimental solution after use. The formaldehyde in the protein immediately after washing was estimated and in another series of experiments the protein was dried for 6 days at 70 per cent. relative humidity before the estimation of the formaldehyde was carried out. In both series the protein was acidified with 40 c.c. *N* sulphuric acid and the formaldehyde was driven off by steam distillation through a condenser into a water-cooled flask. It was found that in order to recover all the formaldehyde it was necessary to collect 500 c.c. of distillate. After well mixing the first 500 c.c. of distillate, 100 c.c. was placed in a stoppered flask and mixed with 25 c.c. *N* sodium hydroxide and 20 c.c. *N*/5 iodine. After one minute 40 c.c. *N* sulphuric acid were added and after a further 5 minutes the excess of iodine was back-titrated with *N*/10 thiosulphate. Standard *N*/10 thiosulphate was prepared and the concentration of the iodine solution was checked frequently, following the above technique. The amount of formaldehyde recovered from the protein was calculated from the volume of iodine which had combined with the formaldehyde, 1 c.c. of 0.2 *N* iodine solution being equivalent to 0.003 g. of formaldehyde:

$$HCHO + I_2 + H_2O = 2HI + HCOOH$$

The moisture content of the collagen, hair and silk was determined by drying to constant weight in a vacuum oven at 100°C., and all results have been expressed on the weight of protein after drying.

(ii) Combination in Unbuffered Solutions.

In this series of experiments, the pH values of the solutions were adjusted by means of hydrochloric acid and sodium hydroxide. Both initial and final pH values were determined, but when representing the results graphically only the final pH values have been considered. Since the solutions were unbuffered the pH values shifted appreciably towards the iso-electric point of the protein except in strongly acid or alkaline conditions.

The amounts of formaldehyde present in the undried proteins are shown in Fig. 2. These figures will obviously include the formaldehyde in chemical combination with the protein, together with any which may be dissolved in the water present in the interfibrillary spaces.

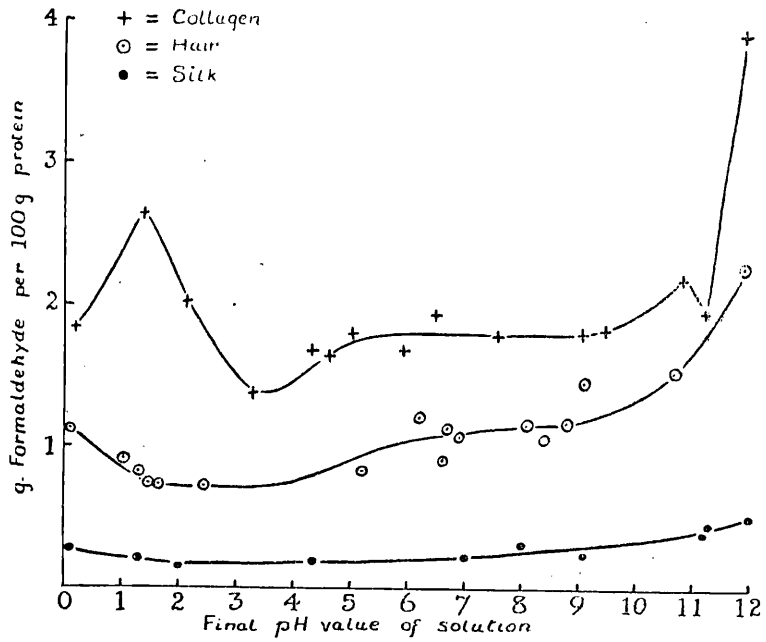


Fig. 2

Unbuffered solutions—wet proteins

It can be seen from the graph that the silk has only taken up a small amount of formaldehyde, even after treatment at high pH values. Between pH 7 and 12, however, the amount of formaldehyde taken up does increase slightly. Hair, after treatment at low pH values, takes up more formaldehyde than silk and above pH 4, the amount taken up gradually increases until at pH 12, 100 grams hair takes up 2.2 grams formaldehyde, while 100 grams silk only takes up 0.5 grams. In the case of collagen there is a well defined maximum in the curve at about pH 1.5 followed by a minimum at pH 3.5. From pH 3.5 to pH 5 the curve rises, after which it is fairly flat, until at pH values above 11 the curve rises steeply. This curve is similar in shape to the corresponding swelling curve, though the minimum in the curve for the amount of formaldehyde taken up by collagen lies in the region of pH 3.5, while the maximum on the acid side lies at about pH 1.5. These values are appreciably lower than the respective minimum and maximum swelling points. The depression in the curve which occurs at about pH 11 is probably real since a similar depression also occurs in the swelling curve (Jordan Lloyd, Marriott and Pleass, 1933). The samples of collagen which had been treated in solutions at pH values between 0.5 and 2.5 were obviously plumped. This fact, in conjunction wtih the resemblance between the combination and swelling curves suggests that in addition to the formaldehyde combined with the protein, there is also a considerable amount of formaldehyde present in solution in the fluid retained by the interfibrillary spaces of

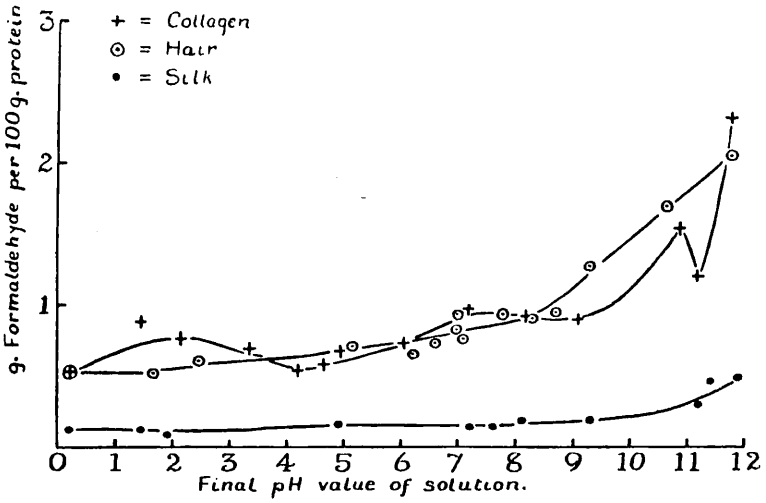


Fig. 3

Unbuffered solutions—dried proteins

the collagen. The greater the degree of plumping, the greater the amount of formaldehyde which will be held in this way.

Hair undergoes little plumping (Jordan Lloyd and Marriott, 1934) and hence the apparent increase in the formaldehyde taken up at low pH values is only slight Apart from this difference collagen and hair behave rather similarly, but the actual amount of formaldehyde taken up by the former is greater at all pH values. At pH 11.8, collagen takes up 3.9 per cent. formaldehyde, as compared with about 2.2 per cent. in the case of hair.

The quantity of formaldehyde retained by the proteins after drying for 6 days at 70 per cent. relative humidity is shown in Fig. 3. One would expect that most of the formaldehyde which was only dissolved in the fluid in the interfibrillary spaces of the wet proteins would volatilise on drying and hence the formaldehyde which has been estimated in the dry proteins may be considered to be chemically combined. The difference between the corresponding curves in Fig. 2 and Fig. 3 represents the amounts of formaldehyde which were not combined with the wet proteins. Silk only absorbs a small proportion of water at any pH value (Jordan Lloyd and Marriott, 1933). Hence, as would be expected, the loss of formaldehyde on drying is very small and the curves for silk in Figs. 2 and 3 are almost identical. The loss of formaldehyde during the drying of the hair was greater than the loss from the silk, but appreciably less than the loss from the collagen. This is in accordance with what would be anticipated from the fact that the water absorption of hair is slightly greater than that of silk but considerably less than that of collagen. The curves for the formaldehyde retained by hair and collagen after drying lie very close together, indicating that the amount combined by these two proteins is similar, but the amount of fluid containing formaldehyde held in the interfibrillary spaces of the wet protein is much greater in

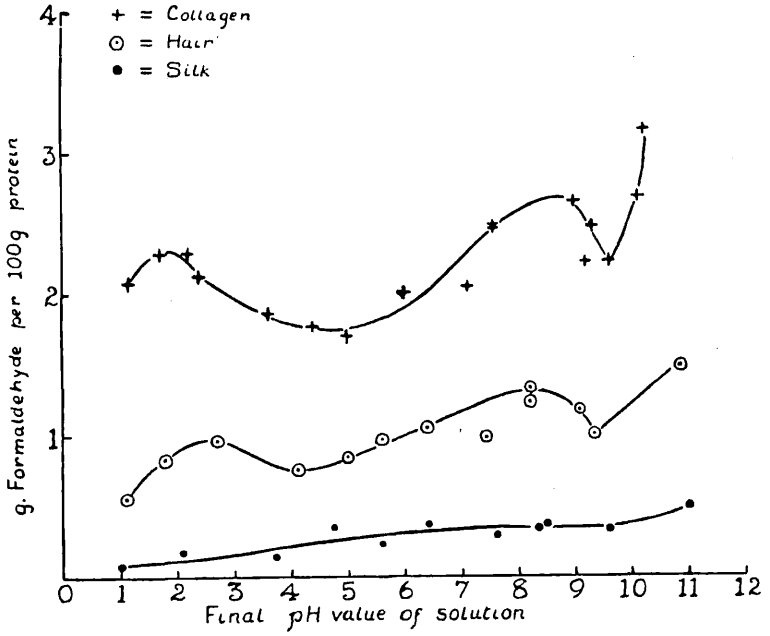


Fig. 4

Solutions buffered with ' Universal Buffer '—wet proteins

the case of collagen than hair. At pH values below 8 the gradients of the curves for dry hair and collagen are low but indicate that even in very acid conditions some formaldehyde is fixed. (At all pH values below 8, less than 1 g. formaldehyde is fixed per 100 g. protein, while at pH values below 1 only 0.5 g. formaldehyde is fixed). Above pH 8 the increase in the amounts of formaldehyde taken up with increasing pH values of the solutions is more rapid in the case of hair than in the case of collagen, and it is not until pH 11.8 is reached that the collagen again fixes as much formaldehyde as hair, namely, about 2 grams formaldehyde per 100 grams protein.

At pH values between 4 and 11, the collagen dried out in a condition similar to that of the acetone dehydrated hide before treatment, while at pH values above 11 the grain tended to become cracky. At pH values below 4 the collagen dried out in a hard and horny condition suggesting that the collagen was not tanned.

(iii) Combination in Solutions Buffered with Universal Buffer.

The same procedure was followed as in the first series of experiments except that the solutions were buffered with The British Drug Houses' " Universal Buffer." This buffer consists of a mixture of phenyl acetic, phosphoric and boric acids. The pH values of the solutions were adjusted by means of hydrochloric acid and sodium hydroxide. The amounts of formaldehyde fixed by the proteins after treatment at pH values ranging from 1 to 11 are represented graphically in Figs. 4 and 5. Fig. 4 gives the values

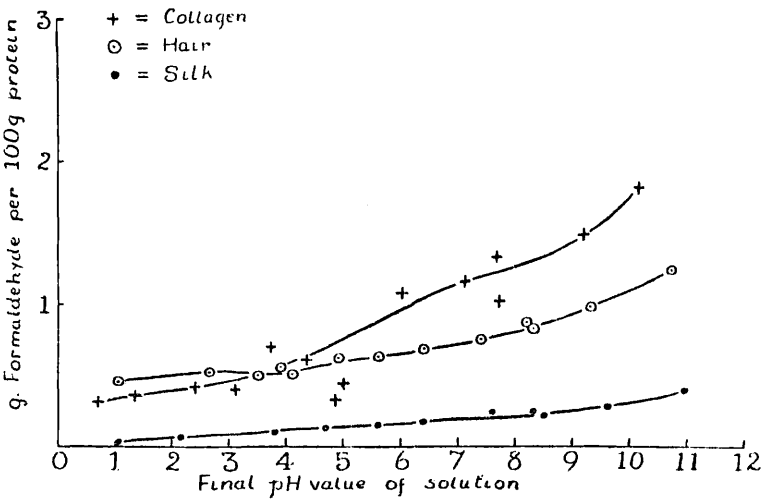


Fig. 5

Solutions buffered with ' Universal Buffer '—dried proteins

obtained after washing only and Fig. 5 the amounts of formaldehyde present after washing and drying out for 6 days at 70 per cent. relative humidity.

The curves for silk in both Figs. 4 and 5 are similar to the curves for silk in Figs. 2 and 3 and indicate that, as in the unbuffered solutions, in the presence of " Universal Buffer " silk only combines with a small amount of formaldehyde. At pH 11, 100 g. silk combines with about 0.4 g. formaldehyde, but as the pH value is reduced the amount of formaldehyde combined is also reduced until at pH values below 2 the amount is almost negligible.

The curves relating to hair in Figs. 2 and 4 show that fairly similar amounts of formaldehyde are taken up in unbuffered and in buffered solutions, but the curves for collagen present more marked differences. As has already been discussed the curve for formaldehyde retained by the wet collagen is influenced to a marked extent by the amount of solution absorbed. The amount of formaldehyde retained but not combined with the wet collagen may be ascertained by comparison of curves on Figs. 4 and 5. The curves for the dried hair in Figs. 3 and 5 are similar below pH 8, but hair treated in buffered solutions contains slightly less formaldehyde than that treated in unbuffered solutions at pH values above 8.

The curves for collagen on Figs. 3 and 5 indicate that in acid solutions collagen combines with rather less formaldehyde in the presence of Universal Buffer, while above the isoelectric point collagen combines with more formaldehyde in the presence of Universal Buffer than in unbuffered solutions. At pH 10, 100 grams collagen treated in buffered solutions combines with 1.8 grams formaldehyde while in unbuffered solutions it only combines with 1.2 grams.

With regard to the condition of the collagen after treatment it was found that at pH values below 4 the collagen was very much plumped and dried

out hard and horny. Above pH 4, in no case was the grain found to be cracky, even after treatment in the most alkaline solutions.

(iv) Combination in Solutions Buffered with Sodium Carbonate.

In this series of experiments the same concentration of formaldehyde was used as in the previous experiments, but solutions containing varying amounts of sodium carbonate, viz., 0, 0.025, 0.05, 0.075, 0.1, 0.125, 0.15, 0.25, 0.5, 1.0 and 1.5 per cent. Na_2CO_3, were used. As before, the final pH values of the solutions after use have been plotted against the amount of formaldehyde estimated in the undried and in the dried protein (Figs. 6 and 7).

The curves indicate that in the presence of sodium carbonate silk only combines with a small amount of formaldehyde, and the slight differences in the curves for the dried and undried silk indicate that a negligible amount of formaldehyde is held in the interfibrillary spaces in the wet protein. The pH value of the solution has only a slight influence on the amount of formaldehyde combined, but the amount combined does increase slowly with increase in pH values. The curves are similar to those for silk in the two systems studied previously.

The curves for hair shown in Figs. 6 and 7 indicate that as the pH rises with increasing concentration of sodium carbonate the amount of formaldehyde fixed increases, but the increase is not marked until the pH value rises above 10. Collagen takes up considerably more formaldehyde and combines with slightly more formaldehyde than hair. The curves for both proteins are similar in general shape, but in the case of collagen the marked increase in fixation of formaldehyde occurs at pH values above 9. There is, however, an irregularity which occurs in both the curves for collagen at about pH 10.5.

On comparison of the curves for the amount of formaldehyde taken up and combined in the sodium carbonate systems with the corresponding curves for the two systems previously studied, it will be observed that at low pH values (below about pH 9), the effect of the sodium carbonate is to decrease the amounts of formaldehyde taken up and slightly to decrease that combined. Above this pH, however, the sodium carbonate tends on the whole to increase the fixation. Since the differences below pH 9 are not so marked in the case of the curves for the dried proteins, it would appear that the sodium carbonate increases the amount of formaldehyde solution held in the interfibrillary spaces to a greater extent than it increases the chemical combination of the formaldehyde with the proteins.

The tendency for the grain layer of the collagen to dry out cracky was most pronounced in this experiment. Using 1.0 per cent. sodium carbonate (final pH value of solution, 10.7) the grain dried out very cracky and it was possible to peel it off. Even using 0.075 per cent. sodium carbonate (final pH value of solution 9.0) there was a tendency to give a cracky grain.

Discussion.

The systems under consideration consist of organised proteins, collagen in the form of pieces of acetone dehydrated ox hide freed from other proteins, hair or silk fibres. The experimental material was immersed in aqueous solutions containing formaldehyde. On immersion the proteins rapidly absorb

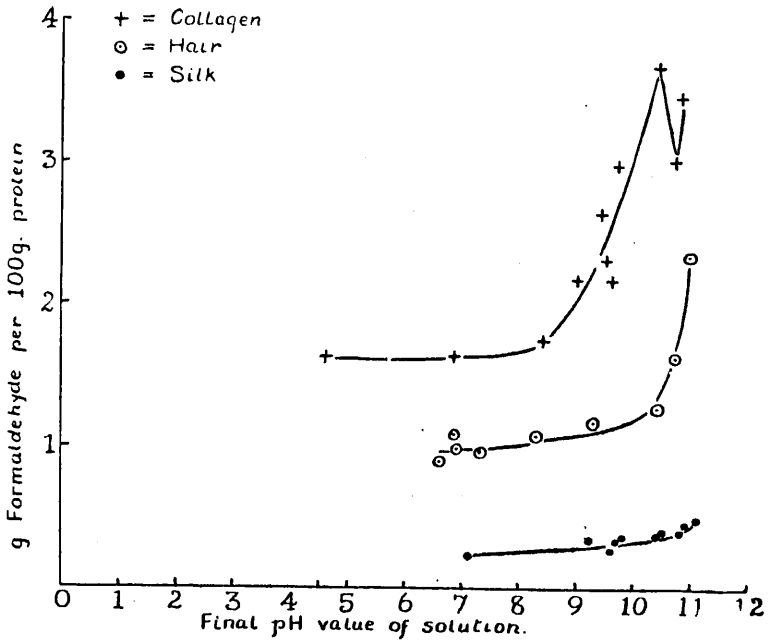


Fig. 6

Solutions buffered with sodium carbonate—wet proteins

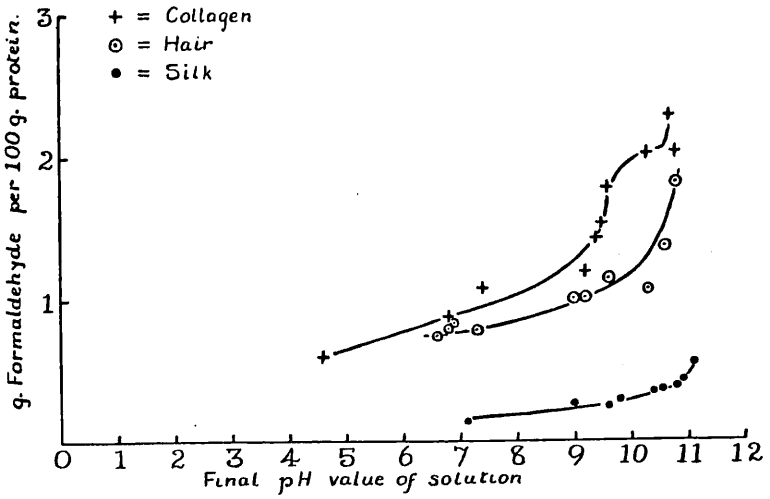


Fig. 7

Solutions buffered with sodium carbonate—dried proteins

the solution into their capillary spaces, and the solution then slowly diffuses into their fine structures where formaldehyde becomes available for combination with the various reacting groups in the protein molecules. Thus, the attainment of equilibrium depends on the rate of diffusion of the solution into the fine structures of the proteins, and also on the rate of reaction of formaldehyde with the various chemical groups involved. It is probable that equilibrium is only reached after prolonged treatment, and it is unlikely, although appreciable amounts of formaldehyde remained in solution, that the reaction was complete at the end of 2 days. Moeller (1921) has reported that hide powder continues to absorb formaldehyde over a period of 3 months and although criticism has been levelled at his technique it is probable that this reaction does continue over a long period of time.

When considering the results of this investigation, therefore, it should be borne in mind that any differences observed may be due, either to an absolute difference in the total amount of formaldehyde which can be fixed in any given conditions, or may be due to an alteration in the rate at which formaldehyde can be fixed, which is determined both by the rate of diffusion and also by the rate of reaction with each particular chemical group.

Hence a complex system is under consideration and insufficient data is at present available to explain all the experimental observations.

So far this investigation has given certain results which may be summarised in the following manner:—

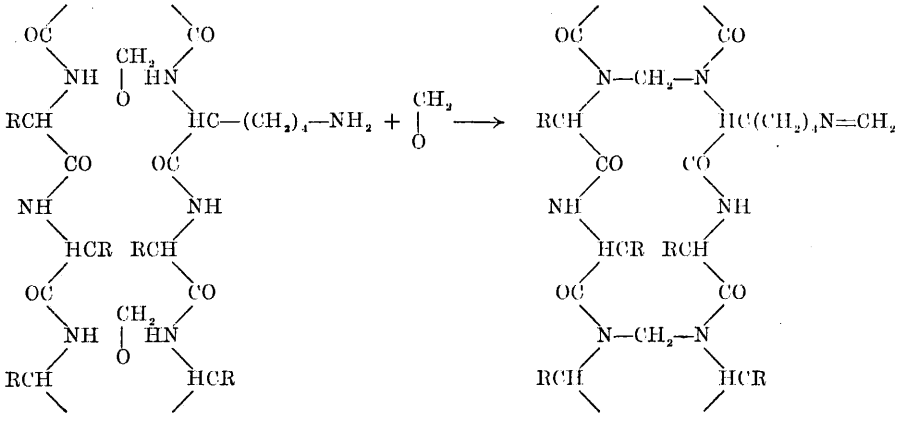
Before drying, the treated proteins contain formaldehyde in combination and also formaldehyde in solution in the interstices of the protein. The amounts of formaldehyde held in these two ways can be differentiated by comparing the amount of formaldehyde present before and after drying the protein. Speaking generally, collagen combines with rather more formaldehyde than hair and in turn hair combines with appreciably more formaldehyde than silk in similar experimental conditions.

Collagen, hair and silk fix some formaldehyde at all pH values, but the higher the pH value of the solution, the greater the amount of formaldehyde fixed by all three proteins. With silk, however, the influence of the pH value on the fixation of formaldehyde is less than in the case of hair or collagen.

The presence of either sodium carbonate or Universal Buffer influences the fixation of formaldehyde. Since there is no evidence of equilibrium having been reached it is not possible to make exact calculations as to which of the reactive groups have combined with formaldehyde under these particular conditions. Obviously, if all the possible groups, i.e., amino, imino (including peptide imino) and sulphydril completely entered into combination far more formaldehyde would be taken up than has been found experimentally. Therefore, either only certain groups are entering into the reaction or else the reaction is not proceeding very far. It is generally considered that the reaction between formaldehyde and amino groups in solution, as in Sörensen's method of formal titration, is rapid and in formaldehyde tanning Stiasny (1936) postulates a rapid reaction with the amino groups followed by a slower reaction with the more numerous peptide groups.

From the curves on Fig. 3 it can be seen that silk combines with much less formaldehyde than does collagen or hair. Table II shows that silk contains very few free amino groups while collagen and hair have considerably more. This also lends support to the theory that the amino groups are playing an important part in determining the quantity of formaldehyde which is fixed by the protein. It has already been shown in Section 1 that the amino groups would be expected to combine more readily with formaldehyde at higher pH values, and the fact that all the curves indicate that at higher pH values hair and collagen, and to a lesser extent silk, fix more formaldehyde than at low pH values is in accordance with combination at the amino groups having occurred to a considerable extent.

Since, however, some combination does occur even in acid solutions below pH 4 where the amino groups would not be expected to react to any appreciable extent, it is possible that combination is occurring at other groups. Alternatively, the salt formation at the amino groups in acid solution may merely reduce the rate of reaction with formaldehyde but not prevent it. Küntzel (1937) suggests that formaldehyde may form a bridge between adjacent protein backbones, combination occurring at the peptide groups as shown below.



Work is at present in progress in order to discover the effect of deaminating collagen on the subsequent fixation of formaldehyde over a range of pH values. This should give an indication of the extent to which the amino groups of the lysine residue are entering into the reaction.

The reason why the addition of salts influences the fixation of formaldehyde by proteins is at present obscure, but it is hoped, by a study of systems containing salts other than sodium carbonate and Universal Buffer, to obtain further data on this point.

From the aspect of practical tanning this work has shown that the optimum pH value at which to aim probably lies between pH 8 and 10. At pH values below 8 little fixation of formaldehyde occurs while at pH values above 10, where considerably more formaldehyde is fixed, a type of case-hardening effect might be expected. Crackiness of grain has been observed at high pH values in unbuffered solutions and in solutions containing sodium

TABLE II

	Parts per 100 parts dry protein			
	Gelatin (Dakin)	Hair (Argiris) (Vickery and Leaven-worth)	Silk (Vickery and Block)	Collagen (High-berger)
Lysine	5·9	1·1	0·2	4·02
Free amino nitrogen	0·57	0·11	0·02	0·39
Formaldehyde equivalent of free amino nitrogen	1·22	0·24	0·04	0·83
Arginine	8·2	7·6	0·7	7·46
Free amino nitrogen	0·66	0·61	0·06	0·60
Formaldehyde equivalent of free amino nitrogen	1·41	1·31	0·13	1·29
Total formaldehyde which can combine with the amino groups	2·63	1·55	0·17	2·14

carbonate. This is probably due to excessive fixation of formaldehyde, particularly at the surface of the grain. In the presence of Universal Buffer no crackiness was observed and a number of workers [Meunier (1912), Gerngross and Gorges (1926), Thomas, Kelly and Foster (1926), Theis and Schaffer (1936)] have suggested that the addition of salts prevents crackiness of the grain.

In order to facilitate the stabilisation of the pH value it is desirable to work with liquors which are well buffered. While sodium carbonate is suitable as a buffer, it does not prevent crackiness of the grain, and, therefore, some other salt should be added as well. The crackiness referred to above is crackiness of the grain of hide which had been limed and from which the hair and epidermis had been removed. Crackiness is also common on skins which have been formaldehyde tanned in the hair or wool and which have the epidermis, which is keratinous in nature, still intact. Comparison of the curves for collagen and hair show that the presence of either Universal Buffer or sodium carbonate causes hair to take up less, and collagen more, formaldehyde than in unbuffered solutions. It is possible that if crackiness of the grain of skins in the wool or hair were due to excessive formaldehyde tanning of the epidermis, that the presence of salt in the tan liquor would prevent crackiness on these types of skins also.

The authors wish to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper, which appeared in its original form in the Laboratory Reports of the Association, 1937.

Summary.

1. The combination of formaldehyde with collagen, hair or silk in unbuffered solutions and also in the presence of Universal buffer solution and sodium carbonate over a wide range of pH values has been studied.

2. Less formaldehyde is taken up by silk than by hair or collagen.

3. With all three proteins greater fixation occurs at high pH values than at low, but this increase is least marked in the case of silk.

4. The presence of the salts studied does not appreciably affect fixation of formaldehyde by silk, increases the fixation by collagen, but decreases the fixation by hair.

5. Formaldehyde tanning is considered to involve some combination of the formaldehyde with the free amino groups, the imino groups and the sulphydril groups in the case of keratins, but the extent to which each of these groups is reacting is not known.

6. The results of this investigation suggest that formaldehyde tanning should be carried out at pH values between 8 and 10. Buffered solutions facilitate the control of the pH value, while the presence of salts helps to prevent cracky grains.

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FORMALDEHYDE TANNING.

PART II.

The Combination of Formaldehyde with Collagen and Keratin (Hair) in the presence of Calcium Hydroxide.

PART III.

The Combination of Formaldehyde with Deaminated Collagen and Keratin.

By

JOANE H. BOWES and WINNIFRED B. PLEASS.

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August, 1939.

FORMALDEHYDE TANNING.

Part II.—The Combination of Formaldehyde with Collagen and Keratin (Hair) in the presence of Calcium Hydroxide.

By Joane H. Bowes and Winnifred B. Pleass.

(Reprinted from the *J.I.S.L.T.C.*, 1939, **23**, pp. 451 to 461).

The combination of formaldehyde with collagen, keratin and silk fibroin in the presence of sodium hydroxide, Universal Buffer and sodium carbonate has already been studied in an earlier investigation (Bowes and Pleass, 1939). This work has now been extended to include calcium hydroxide. It has been suggested that the presence of calcium, even in small amounts, may influence the combination of formaldehyde with collagen. In view of this fact it was decided to determine the amount of formaldehyde fixed by collagen and hair in the presence of calcium hydroxide over a range of pH values.

The experimental procedure was similar to that employed in the previous investigation. A solution of calcium hydroxide was prepared by the careful slaking of quicklime, and after allowing the excess calcium hydroxide to settle the supernatant liquor was poured off. Formaldehyde was added to the calcium hydroxide solution to give a concentration of 0.8 grams per 100 c.c. 5-gram samples of collagen or hair were treated with 200 c.c. of solution adjusted by the addition of $N/10$ hydrochloric acid to various initial pH values ranging from 1 to 12. The reaction was allowed to proceed for 48 hours

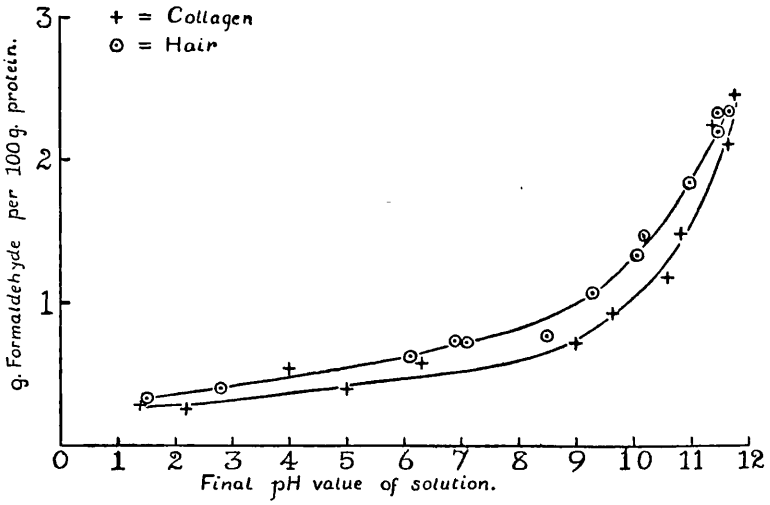

Fig. 1

at room temperature, and the initial and final pH values were recorded. The proteins were then washed and dried and the formaldehyde estimated as described in the earlier paper.

The results have again been presented graphically, the amount of formaldehyde combined being plotted against the final pH value of the solution. The amounts of formaldehyde present in the dried proteins are shown in Fig. 1. For convenience the curves for the combination of formaldehyde with collagen and hair in the presence of sodium hydroxide are again reproduced (see Fig. 2). From Fig. 1 it can be seen that increase in the pH value causes an increase in fixation of formaldehyde by both collagen and hair. This

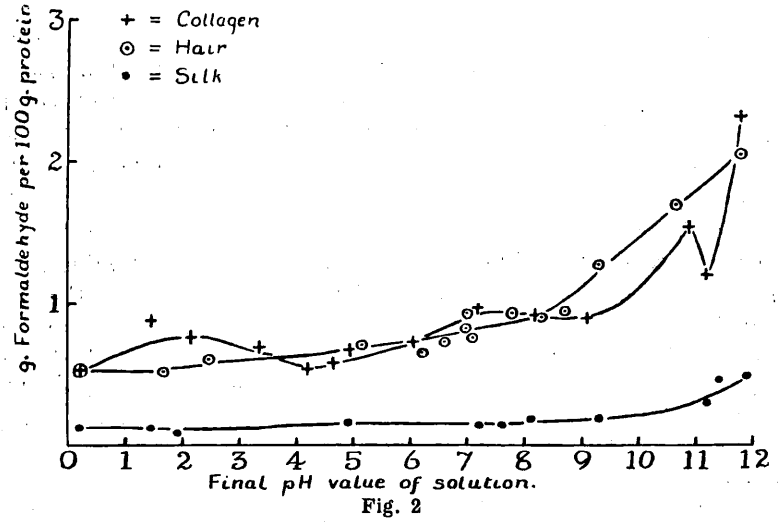

Fig. 2

increase is only slight up to pH 9, but at higher pH values the slope of the curves becomes appreciably greater.

At pH values below about 6.0 the curves for hair and collagen lie very close together, but above pH 8 hair fixes slightly more formaldehyde than collagen. Comparison with Fig. 2 shows that at all pH values, approximately the same amount of formaldehyde is combined with the collagen and hair in both systems, although there is perhaps a slight tendency for less formaldehyde to be fixed in the presence of calcium hydroxide than in the presence of sodium hydroxide.

It may be concluded that the combination of formaldehyde with collagen and keratin is not materially affected by the substitution of calcium for sodium hydroxide.

Reference.

Bowes and Pleass (1939), *J.I.S.L.T.C.*, **23**, 365.

The authors wish to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper, which has been reprinted from the Laboratory Reports of the Association, 1938.

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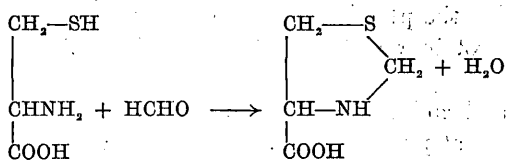
Part III.—The Combination of Formaldehyde with Deaminated Collagen and Keratin.

By Joane H. Bowes and Winnifred B. Pleass.

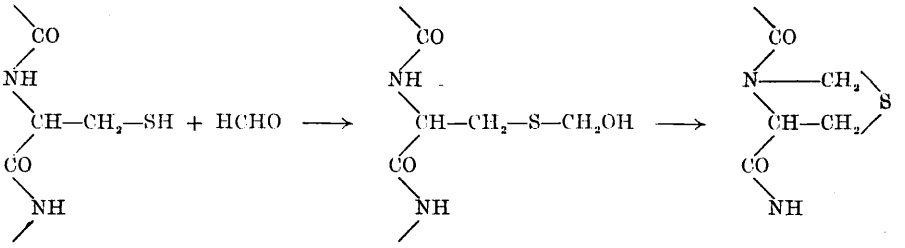
Introduction.

The combination of formaldehyde with collagen, hair and silk was studied in an earlier investigation (Bowes and Pleass, 1939) and suggestions were put forward as to the possible mechanism of the combination. In the case of collagen, the amount of formaldehyde taken up can be accounted for by combination with the free amino groups of lysine and arginine, although the possibility of combination taking place at the imino groups of the backbone is not excluded.

Hair, however, presents a more complex problem. This protein contains only 2 to 3 per cent. of lysine as compared with about 6 per cent. in collagen, yet at similar pH values it was found to fix as much formaldehyde as collagen. To account for this increased uptake of formaldehyde it was suggested that the cystine group present in hair was playing a part in the reaction. Evidence in support of this theory was found in the work of Ratner and Clarke (1937) on cysteine. These workers have shown that formaldehyde combines with cysteine in the following way:—



The reaction first takes place at the sulphydril group and is then followed by ring closure. In the presence of alkaline formaldehyde, the cystine in hair will probably be reduced to cysteine, and so be able to combine with formaldehyde in the following manner:—



This reaction may be followed by ring closure as with free cysteine.

In order to throw more light on the nature of the combination and the part played by the amino groups, it was decided to deaminate samples of collagen and hair and to estimate the formaldehyde taken up by the deaminated proteins. In deamination with nitrous acid the free amino groups of the lysine residues are replaced by hydroxy groups but the free amino groups of the arginine residues are not attacked except under very extreme conditions. If the amino groups are mainly responsible for the fixation of formaldehyde, it would be expected that the deaminated proteins would take up less formaldehyde than the untreated proteins, the decrease being proportional to the lysine content of the proteins.

Experimental Method.

The experimental material (acetone dehydrated collagen and goat hair) was similar to that used in the previous experiments. The proteins were deaminated according to the method used for hide powder by Thomas, Kelly and Foster (1926). 100 g. of the protein were placed in a vessel of 2-litre capacity and 100 g. of sodium nitrite and 2 litres of water added. Carbon dioxide was bubbled slowly through the solution and when the air had been replaced by this gas, 100 c.c. glacial acetic acid were added by means of a thistle funnel. The reaction was carried out in an atmosphere of carbon dioxide in order to prevent undue oxidation of the nitrous acid to nitric acid. Deamination was continued for 48 hours, during which time a further 100 g. sodium nitrite and 100 c.c. glacial acetic acid were added.

Owing to the bulky nature of hair it was only possible to deaminate 50 g. of this protein in one operation.

After 48 hours the solutions were poured off and the proteins well washed in running water. In order to facilitate the removal of acid, the protein was placed in a concentrated salt solution overnight. Washing was continued for 2 to 3 days until the pH of the solution in contact with the protein was about 7.0. The protein was then dehydrated in acetone and stored in a stoppered bottle.

The deaminated collagen and hair were treated with formaldehyde in unbuffered solutions at pH values ranging from 1 to 12. The proteins were

dried at a controlled humidity of 70 per cent. relative humidity for one week, after which the formaldehyde combined with the proteins was estimated.

The experimental procedure throughout was the same as that employed in the previous investigation (Bowes and Pleass, 1939). Some difficulty, however, was encountered in estimating the formaldehyde present in the deaminated hair. The end point of the back titration with thiosulphate was difficult to determine owing to the slow reappearance of the colour due to the liberation of iodine. The cause of this trouble was traced to the presence of nitrites in the formaldehyde distillate, probably derived from the deamination process. It was, therefore, necessary to remove the nitrites from the distillate before carrying out the titration. This was done by means of oxidation with potassium permanganate; 100 c.c. of the distillate were treated with 1 c.c. 10*N* sulphuric acid and 1 c.c. *N* $KMnO_4$ and left for 20 minutes. At the end of this period the residual permanganate was reduced by the addition of 1 c.c. 2 per cent. potassium oxalate and the formaldehyde estimation carried out in the normal manner.

A preliminary experiment, carried out on a solution of known formaldehyde concentration, showed that the above procedure was satisfactory and had no effect on the formaldehyde estimation. The formaldehyde in a given solution was estimated in the usual manner and found to be 1.78 g. per 100 c.c. 400 c.c. of this solution were removed, a small quantity of sodium nitrite added and the solution made up to 500 c.c. 25 c.c. aliquot portions of this solution were treated for removal of nitrite as above and the formaldehyde estimated. The formaldehyde present after this treatment was found to be equivalent to 1.76 g. per 100 c.c. of the original solution, a value in good agreement with that obtained before the addition of nitrite.

The moisture content of the collagen and hair was determined by drying to constant weight at 100°C. and all results have been expressed on the weight of protein after drying.

Section I.—Collagen.

The amounts of formaldehyde combined with deaminated collagen after drying are shown graphically in Fig. 1. For comparison, the combination curve of formaldehyde with untreated collagen under similar conditions is given on the same graph. It will be seen that deaminated collagen combines with considerably less formaldehyde than does untreated collagen at all pH values. This indicates that the free amino groups of lysine, which are removed during the deamination process, are playing an important part in the fixation of formaldehyde by untreated collagen at all pH values. At low pH values deaminated collagen only fixes a very small amount of formaldehyde (0.1 per cent), but as the pH value rises above 9,0, increasing quantities of formaldehyde are fixed, due probably to reaction with the amino groups of the arginine residues. The almost negligible amount of formaldehyde fixed by deaminated collagen at pH values below 9.0 indicates that the imino groups are playing little or no part in the fixation of formaldehyde under these conditions.

It is interesting to calculate the theoretical amounts of formaldehyde which can be fixed by the free amino groups of lysine and arginine in collagen.

TABLE I

Lysine and Arginine Contents of Gelatin and Collagen and the Equivalent Amounts of Formaldehyde

	Parts per 100 parts dry protein	
	Dakin (1920) (Gelatin)	Highberger (1938) (Collagen)
Lysine	5·9	4·02
Formaldehyde equivalent of free amino nitrogen	1·22	0·83
Arginine	8·2	7·46
Formaldehyde equivalent of free amino nitrogen	1·41	1·29
Total formaldehyde which can combine with the amino groups	2·63	2·14

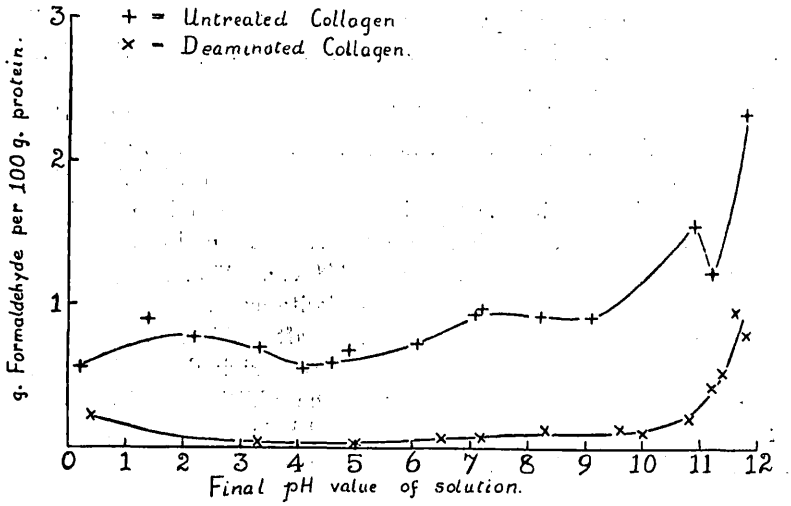


Fig. 1

In Table I are set out the experimental figures of Dakin and Highberger for the amounts of lysine and arginine present in collagen and gelatin, together with the equivalent amount of formaldehyde with which they would be expected to combine. The figures of Dakin are based on gelatin and are, therefore, probably less applicable than the more recent figures of Highberger for collagen.

At pH 9.0, 100 g. untreated collagen fixes about 0.95 g. formaldehyde while deaminated collagen only fixes 0.1 g. under similar conditions. It may be assumed that the difference between these two figures, viz., 0.85 g. per 100 g. collagen, represents the amount of formaldehyde which is combined with the free amino groups of lysine in the untreated protein. This is in agreement with the theoretical amount of formaldehyde which would be expected to be fixed by the lysine groups calculating from the more recent figures of Highberger.

The nitrogen content of the collagen, before and after deamination, was estimated by the Kjeldahl method. The loss of nitrogen was about 0.46 g.

per 100 g. collagen, equivalent to 1.0 g. of formaldehyde, assuming this nitrogen to be present as free amino nitrogen. This figure is in fairly good agreement with the difference in the amount of formaldehyde fixed by the deaminated and the untreated collagen.

It is difficult to give accurate figures for the amount of formaldehyde combined with collagen at high pH values as the curves rise steeply above pH 10. The practical findings, however, are of the same order as would be expected from the figures of Dakin and Highberger for lysine and arginine. The increase in fixation which would be expected at high pH values, due to the amino groups of arginine, is 1.41 per cent. (Dakin) or 1.29 per cent. (Highberger), and it has been found that a rise in the pH value of the experimental solution from 9 to 12 causes an increase in the amount of formaldehyde fixed of the order of 1.0-1.5 g. per 100 g. in the cases of both untreated and deaminated collagen.

These results, as a whole, suggest that it is the amino groups of lysine and arginine which are mainly responsible for the fixation of formaldehyde by collagen. A small amount of formaldehyde may be fixed by the imino groups of collagen but this amount is small compared with the whole.

Section II.—Keratin (Hair).

The amounts of formaldehyde combined with hair and deaminated hair in unbuffered solutions over a range of pH values are presented graphically in Fig. 2. It will be seen at once that deamination has caused a marked decrease in the amount of formaldehyde taken up at all pH values.

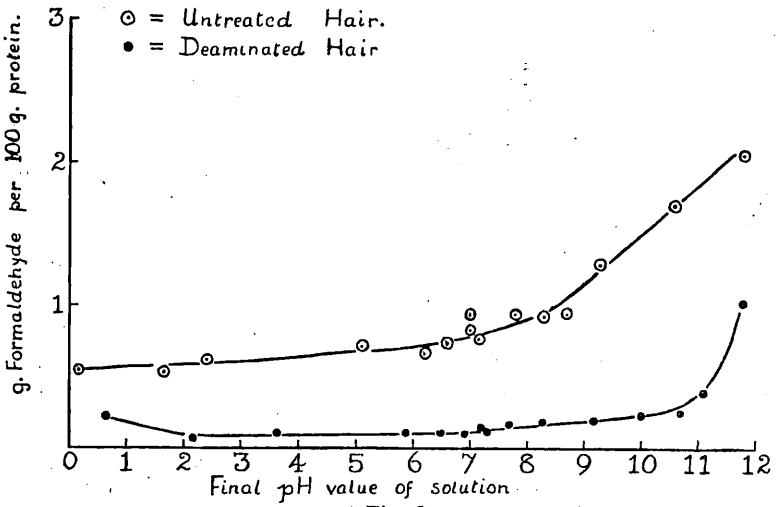


Fig. 2

At all pH values, untreated hair combines with more formaldehyde than can be accounted for by combination at the free amino groups of lysine and arginine only. In Table II the values which have been obtained by various workers for the lysine and arginine content of hair and wool have been summarised. From consideration of these values the maximum amount of formaldehyde which would be expected to combine with hair below pH

TABLE II
Lysine and Arginine Contents of Hair and Wool and the Equivalent Amounts of Formaldehyde

Worker	Date	Type of hair	Parts per 100 parts dry protein			
			Lysine	Formaldehyde equiv. of lysine	Arginine	Formaldehyde equiv. of arginine
Abderhalden and Wells (1920)	1905	Horse hair	1·1	0·23	4·5	0·78
Marston (1928)	1928	Wool	2·8	0·58	10·2	1·76
Vickery and Leavenworth (1929)	1929	Human hair	2·41–2·46	0·50–0·51	5·98–7·96	1·03–1·37
		Horse hair	—	—	7·3–7·6	1·26–1·31
Vickery and Block (1930)	1930	Wool	2·3	0·47	7·8	1·34
Stewart and Rimington (1931)	1931	Wool	2·2	0·45	6·0	1·03

9.0, i.e. with the free amino groups of lysine is only of the order of 0.5 per cent. Up to pH 9.0 hair fixes about 1 per cent. of formaldehyde, indicating the presence of some other grouping in hair capable of combining with formaldehyde.

In collagen it is possible to account for all the formaldehyde fixed by assuming combination at the free amino groups of lysine and arginine. The main difference in the composition of collagen and hair is the presence of the amino acid cystine in the latter. It is, therefore, reasonable to suppose that it is this group which is responsible for the increased fixation of formaldehyde by hair over and above that equivalent to the amino groups of lysine and arginine. The work of Ratner and Clarke (1937), already discussed, on the combination of formaldehyde with cysteine lends further support to this theory.

It would be expected that deamination would cause a decrease in the fixation of formaldehyde proportional to the free amino groups of lysine. This, however, is not the case; up to pH 9.0 deaminated hair fixes about 0.2 per cent. formaldehyde, i.e. 0.8 per cent. less than untreated hair, whereas the formaldehyde equivalent of the lysine amino groups is of the order of 0.5 per cent.; hence some other group capable of reacting with formaldehyde appears to be removed during deamination. This would appear at first sight to run counter to the suggestion that the cystine groups are playing a part in the fixation of formaldehyde by untreated hair. There is some evidence, however, that the cystine groups are attacked during deamination with nitrous acid. Speakman and Stott (1934) have observed that on treatment with nitrous acid more nitrogen is evolved from wool than can be accounted for by the free amino groups and they suggest that this nitrogen is derived from the action of nitrous acid on the sulphur linkage. In support of this, Lough and Lewis (1934) have shown that treatment with nitrous acid causes the sulphur of cystine to be oxidised to sulphate, the presence of which can be demonstrated in the solution. They have estimated the sulphate in the solution

by precipitation with barium chloride and find that up to 85 per cent. of the cystine sulphur may be oxidised to sulphate by prolonged treatment with nitrous acid.

Speakman (1933) states that one cause of perfect elasticity in hair is removed by deamination, and the deaminated fibres are far less liable to take a permanent set than untreated fibres. Since there are few lysine amino groups present in hair, it is probable that some other groups may be involved, possibly the cystine groups.

The general inference which may be drawn from the above evidence is that deamination with nitrous acid causes oxidation of the cystine groups in hair, rendering them incapable of reacting with formaldehyde. The fact that deaminated hair only fixes a small amount of formaldehyde need not, therefore, rule out the possibility of fixation of formaldehyde by the sulphydril groups in untreated hair.

Discussion.

One of the most important points which has come to light as a result of this investigation is the small amount of formaldehyde fixed by the deaminated proteins. This excludes the possibility of the imino groups playing anything but a very minor part in the fixation of the formaldehyde. In formaldehyde tanning, therefore, the action of formaldehyde appears to be confined almost exclusively to the combination at the free amino groups of the arginine and lysine residues. The suggestion of Küntzel (1937) that the formaldehyde may form a bridge between adjacent protein backbones by combining at the imino groups does not, therefore, appear to be likely.

Since the original publication of this paper in the Reports of the British Leather Manufacturers' Research Association circulated privately to members, Highberger and Retzsch (1939), working on the combination of formaldehyde with untreated and deaminated collagen at different pH values, have obtained similar results, from which they have also drawn similar conclusions as follows:—

" (1) In concentrations of 1 per cent. formaldehyde or lower the reaction is confined to the free amino and guanidino groups provided by lysine and arginine respectively.

" (2) One molecule of formaldehyde reacts with each amino and guanidino group.

" (3) The reaction is a molecular one and takes place only with undissociated basic groups.

" (4) In the concentration range noted in (1), only the amino groups of lysine react up to pH 8, above this point the guanidino groups of arginine begin to take part in the reaction.

" (5) The function of the alkaline reaction in promoting formaldehyde tannage is to repress the ionization of the basic groups.

" (6) In formaldehyde concentrations above 1 per cent. an additional reaction begins to come into play in the fixation of larger amounts of formaldehyde. It has been suggested that this may be due either to the extension of the reaction to the imino groups of the protein back-

bone, or to the reaction of the groups already mentioned with polymerized formaldehyde molecules, or to both."

Another interesting point is the combination of formaldehyde with the sulphydril groups in hair. Both experimental and theoretical evidence seem to indicate that it is probable that this reaction occurs to an appreciable extent. If this is so, it is probably a fundamental point and it may be possible to trace some relationship between combination at the sulphydril groups and the occurrence of cracky grain in full grain skins and skins tanned in the wool or hair. In the latter case, the keratin of the epidermal layer is still present and it may be that the combination with the sulphydril groups present is not desirable.

Summary.

1. Collagen and hair have been deaminated with nitrous acid. The formaldehyde fixed by the deaminated proteins at various pH values in unbuffered solutions has been estimated.

2. The combination of formaldehyde with untreated and deaminated collagen is discussed. The formaldehyde fixed by both untreated and deaminated collagen can be accounted for by combination at the amino groups of the lysine and arginine residues only. Deaminated collagen fixes very little formaldehyde, the decrease in the formaldehyde fixed caused by deamination is approximately equivalent to the amino groups of the lysine residues. The imino groups may fix a little formaldehyde but the amount is small compared with the total amount fixed.

3. The combination of formaldehyde with hair and deaminated hair is discussed. At all pH values hair fixes more formaldehyde than can be accounted for by the amino groups of lysine and arginine. It is suggested that the cystine groups present in hair are involved and experimental evidence is brought forward to support this view. Deaminated hair fixes very little formaldehyde, the decrease in fixation due to deamination is greater than that corresponding to the amino groups of lysine. Evidence suggests that the cysteine groups are oxidised during deamination.

4. The practical application of these findings to the tanning process is discussed. It is suggested that the fixation of formaldehyde by the sulphur groupings in the keratin of the epidermis may not be desirable since it may be the cause of the occurrence of cracky grain in skins tanned in the wool or hair.

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EXPERIMENTS ON TREATMENT OF HIDES WITH SOLUTIONS OF WASHING SODA FOR SURFACE DISINFECTION AFTER CONTACT WITH FOOT-AND-MOUTH DISEASE.

By
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(Reprinted from the *J.I.S.L.T.C.*, 1940, **24**, pages 105 to 114).

The general problem of the disinfection of objects contaminated with the virus of foot-and-mouth disease has been investigated by the Foot-and-Mouth Disease Research Committee of the Ministry of Agriculture and Fisheries. It is obvious that many chemical substances and proprietary ' disinfectants ' tested, while they might prove destructive to the virus under certain experimental conditions, would be unsuitable for the treatment of hides. The Committee has given the question of the disinfection of hides special attention. In their Fourth Progress Report[1] they suggested the possibility of employing solutions of sodium bisulphate or sodium bifluoride in certain concentrations for this purpose, but this possibility does not appear to have been pursued further by the Committee. However, O'Flaherty and Doherty (1939) also suggested the use of a solution of sodium hydrogen fluoride for the treatment of hides with a view to destroying the virus of foot-and-mouth disease with which they might be infected. Unfortunately, owing to the difficulties which always beset work with this particular virus, they made their experiments with the virus of *vesicular stomatitis* and not that of foot-and-mouth disease. It will be realised that it is hazardous, especially with infective agents of this nature, to draw conclusions while arguing by analogy. These authors found also that, in certain circumstances difficult to control except under laboratory conditions, the salt was no longer effective against the virus which they were studying.

Moreover, the use of this salt has a disadvantage in that there is a tendency to roughening of the grain of the hide in some instances.

The use of sodium carbonate is discussed at some length in the Fourth Progress Report of the Committee[1] and in the Fifth Progress Report[2] published in 1937 it is stated that, " The methods used to disinfect objects contaminated with foot-and-mouth disease virus have been simplified and reliance abroad (especially in Germany) as in this country is now placed on alkalies such as washing soda."

On occasion and in particular circumstances the Ministry of Agriculture has ordered that hides from cattle which had been in contact with the disease under discussion should be dipped in a 4 per cent. solution of sodium carbonate (washing soda, $Na_2CO_3.10H_2O$).

The Research Association was asked to give its views on this treatment of hides and suggested that members should arrange to have foot-and-mouth disease contact hides immersed in a 4 per cent. solution of common washing soda. A member of the Research Association reported that hides treated with 4 per cent. sodium carbonate ($Na_2CO_3.10H_2O$) solution differed in no way from normal hides, either at the rounding table or after suspenders.

The following investigation was undertaken in collaboration with Messrs. J. & W. N. Hutchings, Ltd., and Imperial Chemical Industries, Ltd., in order to determine whether treatment with 4 per cent. sodium carbonate ($Na_2CO_3.10H_2O$) solution was entirely free from any deleterious effect on the hides.

Works Procedure

For this work, 60 Aberdeen market hides of 60 to 70 lbs. weight were selected. These hides were shaken to remove loose salt and divided into five groups of twelve hides, as indicated in Table I. The hides from one group (Experiment V) received no treatment with sodium carbonate solution and acted as controls. All the other hides were immersed for 15 minutes in

TABLE I

Table giving volume of 4% sodium carbonate solution ($Na_2CO_3.10H_2O$) used for twelve hides for fifteen minutes and the subsequent treatment of the hides

No. of Expt.	Volume of solution	Further treatment
I	280 gallons	I_0 Put into work at once I_1 Stored 1 month I_3 Stored 3 months I_6 Stored 6 months
II	500 gallons	II_0 as in I II_1 ,, II_3 ,, II_6 ,,
III	1000 gallons	III_0 as in I III_1 ,, III_3 ,, III_6 ,,
IV	Once-used liquor from Experiment III	IV_0 as in I IV_1 ,, IV_3 ,, IV_6 ,,
V	No treatment	V_0 as in I V_1 ,, V_3 ,, V_6 ,,

4 per cent. solution, varying ratios of liquor to goods being used for each group (see Table I). The twelve hides in Experiment I were only just covered with solution; the hides in Experiment II were comfortably immersed, while in Experiments III and IV there was a large excess of solution. In Experiments III and IV the goods were handled once during immersion, so that the hides should get the full effect of the large volume of liquor.

The time chosen for immersion was 15 minutes, since this time is generally recommended by the Ministry of Agriculture where only surface contamination is involved.

After treatment the hides were well drained, washed by passing through a water pit and either salted down for storage or put straight into work. Each group of twelve hides was divided into four sub-groups, " 0," " 1,"

" 3 " and " 6." The hides in the " 0 " series of experiments were put straight into work, the hides in the " 1," " 3 " and " 6 " series of experiments were salted down and stored in the salted condition for 1, 3 and 6 months respectively before putting into work.

The hides from all groups were examined during processing at the rounding table, at the head of the first suspenders and in the warehouse, and samples were taken from the official sampling place for microscopical examination. Six-inch square samples were also taken from the finished leather for abrasion tests.

The alkalinity of the used sodium carbonate liquor and of the wash liquors was determined by titration and expressed as percentage of $Na_2CO_3.10H_2O$, in order to determine whether the hides had taken up significant quantities of sodium carbonate during the treatment.

The alkalinity of all the used liquors was very little lower than that of the new liquors, namely 3.9 per cent. $Na_2CO_3.10H_2O$. This suggests that the hides are removing not more than 1 to 2 per cent. of the alkali present; in fact, in the case of Experiment IV, the further immersion of these twelve hides in the used liquor from Experiment III has not appreciably affected the concentration of sodium carbonate.

The sodium carbonate liquors, therefore, could be used, say, four or five times before running to the drain or if made up each time with about 2 per cent. of the original weight of washing soda, they could easily be used ten to twelve times before discarding.

The volume of the sodium carbonate carried away by the hides when lifted out of the pit could not readily be determined but the amount of alkali carried into the wash pit could be gauged to some extent by the analysis of the wash pit liquors. The alkalinity of these rose to over 1 per cent. $Na_2CO_3.10H_2O$ after 48 hides had been through the pit. Part of this was washed off the surface of the hides, but part probably arose from alkali diffusing from the hide.

Works Report on Hides in Process and Finished Leather

Examination of the hides in process and of the finished leather in the warehouse indicated that the treatment with sodium carbonate had had no adverse effect. At the rounding table and at the head of the first suspenders, it was not possible to detect any difference between the control hides and the treated hides in any of the series " 0," " 1," " 3 " or " 6." The hides in Experiments III_3 and V_3 seemed rather plumper in the limed condition than the other hides in this series, but since hides in Experiment V_3 had received no treatment while those in Experiment III_3 had received the most drastic treatment with sodium carbonate, this observation appears to have little significance and may have only been due to the hides being plumper in the first place.

It was felt all through the whole experiment that if the treatment had any effect at all it was a good one.

Microscopical Examination of Samples of Pelt and Finished Leather

(i) Examination of Limed Samples.

The microscopical examination of the samples from hides which had been put straight into work gave little evidence that any damage had occurred through treatment of the hides with sodium carbonate solution under these experimental conditions. The hides in Experiment III_0, which had been treated with the largest volume of sodium carbonate solution, have slightly less full fibres than the hides in Experiment V_0, which had had no carbonate treatment, but in other respects there is little to choose between the microscopical appearance of the samples from these two groups. Fig. 1 shows the fibre structure of one of the control hides (Experiment V_0) while Fig. 2 shows the fibre structure of one of the treated hides in Experiment III_0. The hides, which had been treated in smaller volumes of sodium carbonate solution, and the hides which had been treated in the once-used liquor, are very similar in fibre structure to the untreated hides.

The microscopical examination of the samples from hides, which were stored for one month before putting into work, showed that these samples were similar to those samples which were put straight into work, that is, the only suggestion of any deterioration caused by the sodium carbonate solution is in the samples from Experiment III, where the fibres tend to be slightly thinner than in the other samples.

The samples from hides which had been stored for 3 months before putting into work were examined microscopically and showed that treatment in the small volume of sodium carbonate solution caused no damage to the hides; in fact, the hides in Experiment I_3, which had had this treatment, were slightly better than the controls (Experiment V_3). The samples II_3, which were treated in 500 gallons of sodium carbonate solution for 12 hides, do not show any signs of deterioration, but the samples III_3 and IV_3, which had been treated in the largest volume of sodium carbonate, show some suggestion of slight deterioration. Generally, the fibres are rather thinner and of a lower angle of weave and in some samples the weave pattern is less regular.

The microscopical examination of the limed samples from hides which had been stored for 6 months before putting into work showed that no damage had been caused by the sodium carbonate treatment in Experiments I_6 and II_6, i.e. where the volume of sodium carbonate solution used for 12 hides was 500 gallons or less. In fact, the fibre structure of the samples I_6 is slightly preferable to that of the samples which had had no sodium carbonate treatment. The samples I_6 generally have rather fuller fibres and a more regular weave pattern. The samples III_6 and IV_6, which were treated in 1,000 gallons of sodium carbonate solution have slightly less good microscopical structures than the control samples, the fibres being generally rather thinner and of a slightly lower angle of weave. These differences in fibre structure are shown in photomicrograph, Figs. 3 and 4, which refer to samples from Experiments V_6 and III_6 respectively.

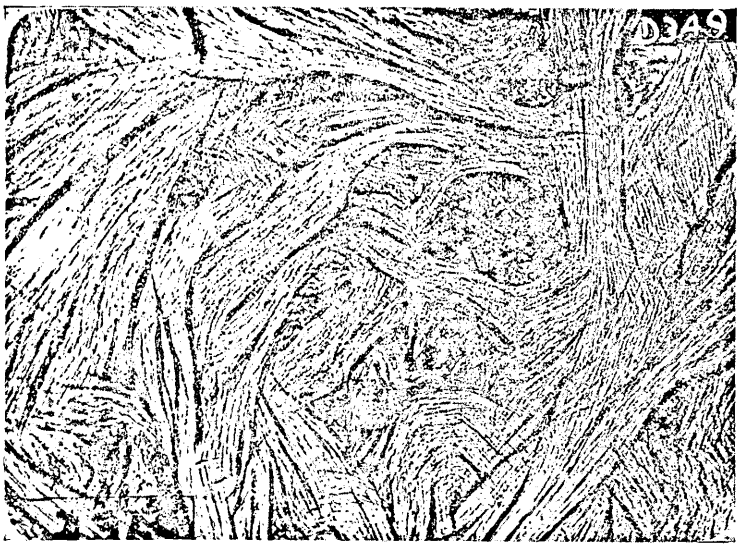

Fig. 1

Experiment V_0.—Limed hide. No sodium carbonate treatment. Hides put straight into work.

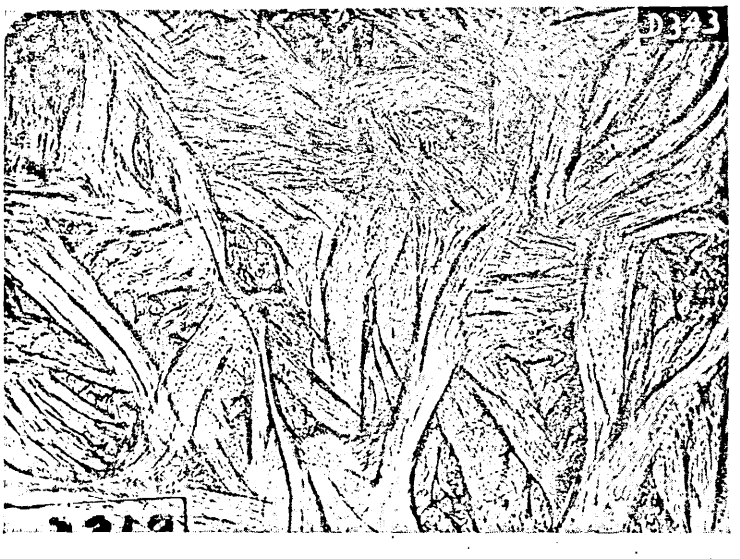

Fig. 2

Experiment III_0.—Limed hide. 1,000 gallons sodium carbonate solution per 12 hides. Hides put straight into work.

The general inference to be drawn from the examination of the limed samples is that treatment of hides in large volumes of sodium carbonate solution (1,000 gallons to 12 hides) may lead to slight deterioration of the hide and this deterioration becomes more noticeable if the hides are stored for some time (3 to 6 months) before putting into work. Treatment of twelve hides in 500 gallons of sodium carbonate solution appears to cause no damage to the hides which can be detected microscopically in the limed condition, whilst treatment in 280 gallons of sodium carbonate solution results in a very slight improvement in the microscopical appearance of the limed pelts. It should be emphasised, however, that the differences in microscopical appearance of any of the limed samples which can be attributed to the sodium carbonate treatment are very small.

(ii) Examination of Samples at the Head of the First Suspenders.

Every experimental hide was sampled at the top of the first suspenders and examined microscopically. At this stage of the process the hides are acid plumped and it is not easy to detect deterioration of the fibre structure caused by any preliminary treatment of the hides, unless the deterioration is fairly marked. As was shown from the examination of the limed samples, any deterioration which had occurred was only slight and, therefore, it is not surprising that the comparison of the samples at the head of the suspenders has been difficult. It can be stated, however, that in no case did the sodium carbonate treatment produce any marked change in the fibre structure of hides so treated and that even those hides which had been treated in the largest volume of sodium carbonate solution and then stored for 6 months, were very little different in microscopical appearance from the control hides which had had no carbonate treatment.

(iii) Examination of Samples of Finished Leather.

Microscopical examination of the finished leather from hides which had been put straight into work or stored for only 1 month, gave no evidence that the immersion in sodium carbonate solutions had affected the condition of the hides in any way.

In the case of hides which had been stored for 3 or 6 months before being put into work, there is some suggestion that the fibre structure of the hides which had been treated in the large volumes of sodium carbonate solution (Experiments III and IV) is not quite as good as that of the controls or the hides from Experiments I and II, the fibre structure of the hides from Experiments III and IV being less compact and of a rather lower angle of weave. This is illustrated in Figs. 5 and 6, which show the fibre structure of hides from Experiments V_3 and III_3 respectively.

There is no evidence that the hides which had been treated in the smaller volumes of sodium carbonate solution and stored for 3 or 6 months (Experiments I_3, II_6, II_3 and II_6) have suffered any damage. In the series which had been stored for 6 months, one of the control hides and one of the hides treated in 280 gallons of carbonate solution have a particularly compact fibre structure and high angle of weave. These two samples also have particularly high resistances to abrasion (see below).

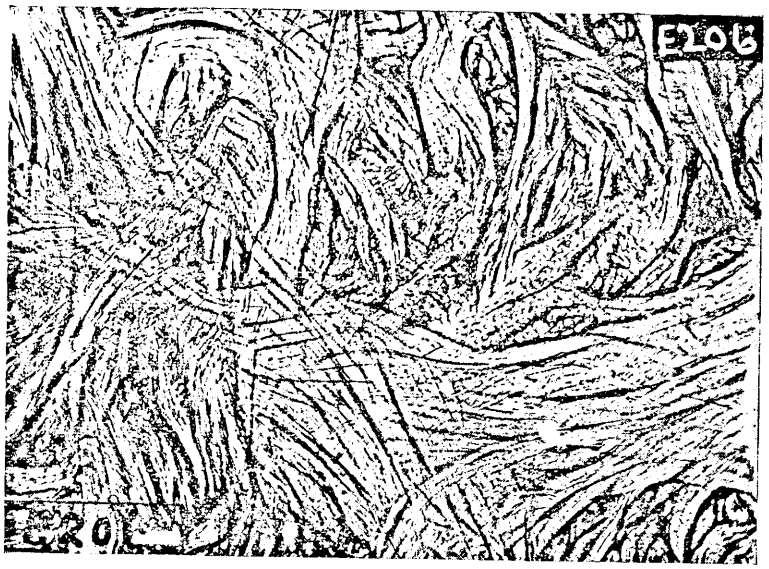

Fig. 3

Experiment V_6.—Limed hide. No sodium carbonate treatment. Hides stored 6 months.

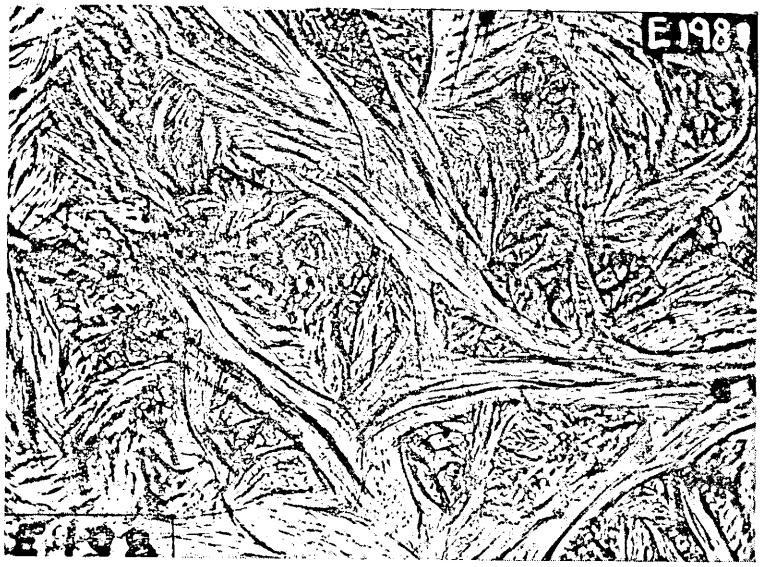

Fig. 4

Experiment III_6.—Limed hide. 1,000 gallons sodium carbonate solution per 12 hides. Hides stored 6 months.

Abrasive Resistance of Samples of Finished Leather

A number of the leathers were examined for abrasive resistance. Since, however, the works report and the microscopical reports on the leather indicated that if any deterioration had occurred due to the soduim carbonate treatment it was undoubtedly slight, it was considered unnecessary to determine the abrasive resistance of all the samples available.

The samples were chosen, therefore, so that the effect of using the largest (Experiment III) and the smallest (Experiment I) volume of sodium carbonate solution could be ascertained both on hides which were put straight into work (" 0 " series) and also on hides which were stored for six months before putting into work (" 6 " series). Control hides which had had no sodium carbonate treatment (Experiment V) were also examined. Two hides were abraded in each instance.

TABLE II

Abrasion Results

Expt. No.	Description	Average abrasive loss (whole thickness) mm. per min.	Average abrasive loss (corium) mm. per min.
I_0	280 gallons	0·430	0·380
	Put straight into work	0·519	0·442
III_0	1000 gallons	0·498	0·430
	Put straight into work	0·344	0·280
V_0	No treatment	0·421	0·381
	Put straight into work	0·395	0·343
I_6	280 gallons	0·245	0·179
	Stored 6 months	0·453	0·348
III_6	1000 gallons	0·272	0·262
	Stored 6 months	0·317	0·255
V_6	No treatment	0·220	0·161
	Stored 6 months	0·494	0·412

The values which have been obtained for the abrasive resistance of the finished leather samples which have been examined are given in Table II. It can be seen from these figures that the values obtained for the duplicate leathers show a wide variation. This, however, is not greater than that commonly found in groups of supposedly similar leathers.

The value obtained for the abrasive resistance of a leather depends partly on the condition of preservation of the raw hide and partly on the conditions of tannage. There is no evidence from the figures in Table II that the carbonate has, in any experiment, lead to an appreciable increase in the rate at which either grain or corium wear away under the action of an abrasive force.

Conclusions

As a result of the experiments described above it may be concluded that the immersion of hides in a 4 per cent. solution of washing soda for 15 minutes at room temperature does not cause any damage to the hides. In

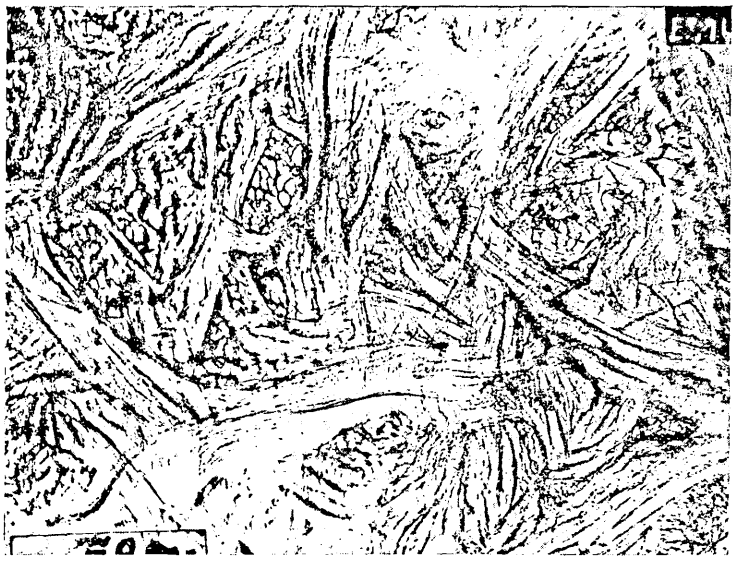

Fig. 5

Experiment V_3.—Finished leather. No sodium carbonate treatment. Hides stored 3 months.

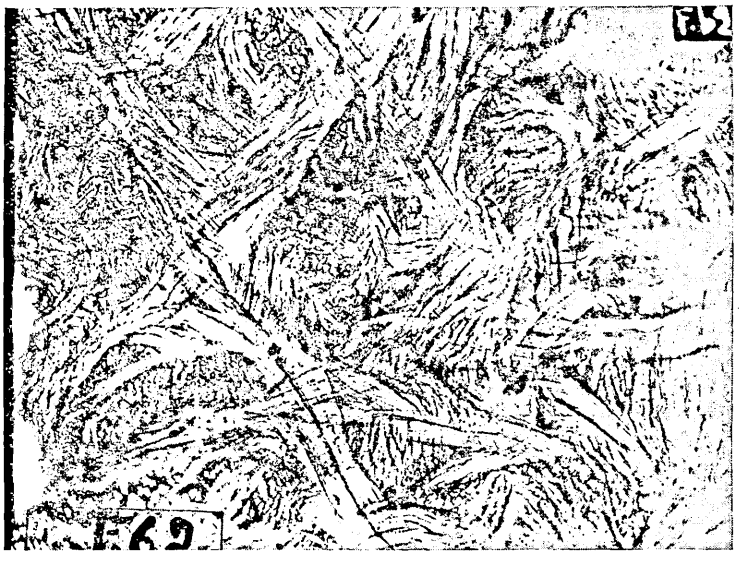

Fig. 6

Experiment III_3.—Finished leather. 1,000 gallons sodium carbonate solution per 12 hides. Hides stored 3 months.

the experiments involving the use of a large ratio of liquor to hides (Experiments III and IV), the microstructure in the limed pelt and in the finished sole bend was slightly less good than that of the control hides or other experimental hides. The deterioration, if any, was not sufficient to lower the figures for abrasive loss or to detract from the appearance of the bends in the warehouse.

Since even the hides which had been treated in the very large volumes of washing soda solution showed no obvious damage it is probably safe to assume that there is a fair margin of safety in this method of treatment. It is probable, therefore, that the treatment could be prolonged beyond the time of 15 minutes without causing any appreciable damage to the hides. If, therefore, owing to particular circumstances attached to any one case of salvage, the hides remain in the disinfecting bath for an hour, it is unlikely that they will suffer any serious harm.

Summary

Solutions of alkaline salts such as sodium hydroxide and sodium carbonate, if sufficiently concentrated, are known to have a destructive action on the virus of foot-and-mouth disease. On occasion and in particular circumstances the Ministry of Agriculture has ordered that hides from cattle which had been in contact with the disease under discussion should be dipped in a 4 per cent. solution of sodium carbonate. The present investigation was undertaken in order to determine whether this treatment was free from any deleterious effect on the hides.

It was found that hides may be treated with sufficient 4 per cent. sodium carbonate solution to ensure complete immersion without having any harmful effect. Although the evidence is not definite that the use of large volumes causes any damage to the hides, it is suggested that it is advisable not to use excessively large volumes of the carbonate solution.

If hides are immersed in a volume of liquor in which they are just comfortably submerged, the disinfecting bath can be used several times without losing much strength.

We wish to thank the Council of the British Leather Manufacturers' Research Association and also Messrs. J. & W. N. Hutchings, Ltd., for permission to publish this paper.

We should also like to express our thanks to Dr. Galloway, of the Experimental Research Station of the Foot and Mouth Research Committee, for the interest which he has taken in this work and the helpful suggestions which he has made.

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D. & L. 1958. 20

THE USE OF AMINES IN THE FELLMONGERING OF SHEEP SKINS AND THE LIMING OF GOAT SKINS.

By

J. H. Bowes and W. B. Pleass.

(Reprinted from the *J.I.S.L.T.C.*, 1942, 26, pages 140 to 145).

The use of amines as unhairing agents has been studied extensively in America and it is now a recognised commercial practice to add mono- or dimethylamines to lime liquors.

McLaughlin and O'Flaherty[1, 2] have carried out experiments on the effect of the addition of amines to lime liquors on the fibre structure of ox hide, and conclude that amines have the same effect on unhairing as sulphide and cause no apparent damage to the hide. Moore, Highberger and O'Flaherty[3] confirm these findings with regard to dimethylamine and monomethylamine; the rate of unhairing was accelerated and no harmful effects such as swelling or loss of hide substance were observed. Trimethylamine, however, they found to be comparatively inactive. The use of dimethylamine for the liming of calf skins has been shown to be satisfactory, the fibre structure of samples treated in lime liquors containing 0.1 per cent. dimethylamine compared favourably with similar samples unhaired in lime-sulphide liquors (Conabere and Merry[4]).

In this investigation a study has been made of the effect of mono-, di- and tri-methylamines on the fibre structure of two types of light skins, i.e., sheep and goat.

The Fellmongering of Sheep Skins.

(i) Experimental Method.

A wool sheep skin which had been pickled for three months was used for these experiments. The skin was depickled in borax for two days and washed in running water for several hours before painting.

In a preliminary experiment, fellmongering pastes of the following composition were made up:—

(1) Lime alone.
(2) Lime + 8 per cent. sodium sulphide crystals (2.6 per cent. Na_2S).
(3) Lime + 1 per cent. monomethylamine,

and in a second experiment:—

(1) Lime.
(2) Lime + 4 per cent. sodium sulphide crystals (1.3 per cent. Na_2S).
(3) Lime + 1 per cent. monomethylamine.
(4) Lime + 2 per cent. monomethylamine.
(5) Lime + 1 per cent. dimethylamine.
(6) Lime + 2 per cent. dimethylamine.
(7) Lime + 1 per cent. trimethylamine.
(8) Lime + 2 per cent. trimethylamine.

Two pieces of sheep skin 4ins. by 6ins. were painted with each paste. After the paint had been allowed to dry slightly the two pieces were placed flesh to flesh and left until the wool could be pulled comparatively easily; this was usually possible after 2 to 4 days. The samples were then dewooled and passed on into a 4 per cent. lime suspension for a further 6 days. At the end of this period sections were cut and photographed. The limed samples were then delimed in 0.5 per cent. ammonium chloride, pickled in sulphuric acid and sodium chloride and chrome tanned. The pieces were dried and examined microscopically.

(ii) Influence of Methylamines on the Rate of Dewooling.

The addition of sodium sulphide or of mono- or dimethylamine to the lime paste increased the rate of dewooling. Using a paste containing lime alone the wool could only be pulled with difficulty after 4 days, while using the lime-sulphide paste containing 4 or 8 per cent. sodium sulphide ($Na_2S.9H_2O$) the wool was quite loose after a few hours. The addition of mono- and dimethylamine increased the rate of dewooling to a lesser extent and it was 2 days before the wool could be pulled easily, the higher concentrations being slightly more effective than the lower concentrations. The paste containing trimethylamine was only a little more rapid in its action than lime alone.

(iii) Influence of Methylamines on the Fibre-Structure.

In the preliminary experiment, the difference in the fibre structure between the three samples was pronounced. The fibre structure of the sample dewooled with lime alone was fairly compact and well opened up, but the fibres were rather thin. The addition of 1 per cent. monomethylamine to the fellmongering paste increased the fullness of the fibres and the section had rather a plumped appearance. This plumping of the fibres was

still more marked in the section of the sample which had been painted with lime and sulphide; the fibres were very full and there was more splitting up apparent. It would appear, therefore, that methylamine has a plumping action on the fibres similar to that of sulphide. The action of the sulphide is the more pronounced but it must be remembered that the concentration of the sulphide in the paint is greater than that of the methylamine. In this experiment the limed samples were not subsequently chrome tanned.

Microscopical examination of the limed samples in the second and more detailed experiment confirms that the fibre structure is affected by the addition of methylamines to the fellmongering paste. The fibre structure of the sample painted with lime alone was rather loose and the fibres were rather thin and not very well opened up. After chrome tanning the fibre structure was still not good, the fibres although fairly full were poorly opened up with many open spaces. The addition of sodium sulphide to the fellmongering paste caused a marked improvement in the fibre structure of both the limed and the crust skins, the fibres were still fairly full, and were better opened up and more regularly woven than in the corresponding samples painted with lime alone.

The addition of 1 per cent. monomethylamine to the fellmongering paste produced a limed pelt rather similar in fibre structure to that produced by the lime-sulphide paste. The fibres, however, were not so full. After chrome tanning the amount of splitting up was considerably greater than in the case of the pelt painted with lime and sulphide. The substitution of dimethylamine for monomethylamine did not produce a pelt of quite such good fibre structure at the limed stage or after chrome tanning. After liming the fibres were fairly well opened up but the angle of weave was rather low. After chrome tanning the angle of weave was still low and the fibre structure was not so evenly and finely opened up as that of the sample painted with a paste containing monomethylamine.

The addition of 1 per cent. trimethylamine had a plumping effect on the fibres and caused slight increase in the amount of splitting up. After chrome tanning, however, the fibre structure of the crust skin was little better than that produced by lime alone.

A greater amount of splitting of the fibres in the limed pelt was observed when the concentration of the methylamines in the fellmongering paste was increased to 2 per cent. and with di- and trimethylamine this increase in splitting up was accompanied by some disorganisation of the weave pattern. After chrome tanning the fibre structure of the three crust skins was not very good, suggesting that the opening up had been carried too far. The fibres were thin and gave the impression of having become stuck together again, especially in the case of the 2 per cent. monomethylamine, where a definite layered effect was visible.

Generally speaking, the results of these investigations indicate that the effect of methylamines on the rate of dewooling and the fibre structure of sheep skins is similar to that of sodium sulphide. Sodium sulphide probably produces the best fibre structure both in the limed and crust state; in fact,

the fibre structure of this crust sample may be considered satisfactory for most purposes. If, however, a greater degree of opening is desired in order to produce a very soft skin, such as those designed for gloving leathers, the presence of 1 per cent. monomethylamine in the fellmongering paste may be advantageous. The use of 1 per cent. dimethylamine in place of monomethylamine is rather less satisfactory, the crust skin being not quite so finely and evenly split up and the general appearance suggesting that some of the fibres have become stuck together again. The addition of 2 per cent. of any of the three methylamines to the fellmongering paste causes rather too much opening up at the limed stage with consequent sticking together of the fibres at the crust stage.

It has been suggested that a particular advantage of the use of methylamines in lime liquors is that the leather produced has a better and smoother grain than leathers produced from skins unhaired with lime and sulphide. Even with these small experimental crust samples it was noticed that the grains of those which had been treated with a methylamine lime paste were much smoother than those which had been treated with either lime alone or a lime and sodium sulphide paste.

The Liming of Goat Skins.

(i) Experimental Method.

A dry-salted goat skin was soaked for three days in a 5 per cent. solution of sodium chloride containing 0.1 per cent. " Chloros." The skin was then washed for several hours in running water and divided into sixteen equal sized pieces. Two samples were then limed in 500 c.c. of each of the following lime liquors:—

(1) 6 per cent. CaO.
(2) 6 per cent. CaO + 0.2 per cent. sodium sulphide crystals (0.06 per cent. Na_2S).
(3) 6 per cent. CaO + 0.5 per cent. sodium sulphide crystals (0.2 per cent. Na_2S).
(4) 6 per cent. CaO + 0.05 per cent. monomethylamine.
(5) 6 per cent. CaO + 0.1 per cent. monomethylamine.
(6) 6 per cent. CaO + 0.05 per cent. dimethylamine.
(7) 6 per cent. CaO + 0.1 per cent. dimethylamine.
(8) 6 per cent. CaO + 0.05 per cent. trimethylamine.

Pieces of pelt were removed for sectioning after 2, 4 and 7 days. At the end of 7 days the samples all unhaired easily. The unhaired samples were delimed in 0.5 per cent. ammonium chloride, pickled in 1 per cent. sulphuric acid and 10 per cent. sodium chloride and chrome tanned. Sections of the skins were cut in the crust state before staking.

(ii) Influence of Methylamines on the Fibre-Structure.

Microscopical examination of the sections of pelt showed that the addition of sulphide or methylamines to the lime liquors has a similar effect on the fibre-structure of goat skins, as their addition to fellmongering paints has on the fibre structure of sheep skins.

The samples treated with lime alone were insufficiently opened up even

after seven days. This lack of opening up in the limed state resulted in a rather poor crust skin, the fibres were thin, poorly opened up, and loosely woven, with an almost horizontal angle of weave. The addition of 0.2 per cent. sodium sulphide to the lime liquor caused an increase in the fullness of the fibres and the amount of opening in the limed pelt at all stages. These features persisted in the crust skin but the weave pattern was not very regular. The addition of 0.5 per cent. sodium sulphide produced a greater plumping effect after 7 days liming than did the addition of only 0.2 per cent. The fibres of both the limed and crust skins were much opened up and the latter had a rather disorderly weave pattern.

The addition of 0.05 per cent. of monomethylamine caused some plumping and some splitting up of the fibres, but the latter had not been carried far enough for most purposes, even after seven days liming. The section of the crust skin showed a slightly layered effect with a horizontal fibre weave. The fibre structure of this sample was little better than that of the pelt treated with lime alone.

The addition of 0.1 per cent. monomethylamine to the lime liquor gave the best results. The fibre structure of the samples of limed pelt after 2, 4 and 7 days appeared to be more opened up and less plumped than that of the corresponding samples limed with the addition of 0.2 per cent. sodium sulphide. The crust leather had what is, for most general purposes, the best fibre structure of the series; the fibres were fairly full, well opened up and regularly woven.

The addition of 0.05 or 0.1 per cent. of di- or trimethylamines to the lime liquor resulted in extensive plumping of the fibres at all stages of liming, unaccompanied by any apparent increase in the amount of splitting up. This resulted in the production of crust skins having a poor fibre structure with thin fibres and a rather disorganised weave pattern. The skin limed in the presence of 0.1 per cent. was slightly worse than the skin limed in the presence of the lower concentration. Possibly a lower concentration of dimethylamine might produce satisfactory results.

A general consideration of these results indicates that the addition of monomethylamine to the lime liquor is mainly effective in causing opening up of the fibre structure together with slight plumping of the fibres. Di- and trimethylamine appear to plump the fibres to a greater degree than monomethylamine without causing any apparent increase in the amount of opening up. This produces a disorganisation of the weave pattern in the crust skin. The addition of 0.1 per cent. monomethylamine to the lime liquor appears to be beneficial, the crust skin produced having a good fibre structure suitable for most purposes.

As with the crust sheep skins, the crust goat skin samples which had been treated with a methylamine lime were much smoother on the grain than those which had been treated with either a sulphide lime or a straight lime.

Summary.

The addition of sodium sulphide or mono- or dimethylamines to the

fellmongering paste increases the rate of dewooling and affects the fibre structure of sheep skins.

Dewooling takes place rapidly in the presence of sodium sulphide (4 or 8 per cent.) $Na_2S.9H_2O$), less rapidly in the presence of 1 per cent. or 2 per cent. mono- or dimethylamines and only comparatively slowly with lime alone or with lime plus trimethylamine.

The addition of sodium sulphide (4 per cent. $Na_2S.9H_2O$) to the fellmongering pastes improves the fibre structure of sheep skins and produces satisfactory limed pelt and chrome crust skin which is probably suitable for most purposes. The addition of 1 per cent. monomethylamine causes a greater amount of splitting up in the crust skin and may therefore be advantageous for certain purposes.

The addition of a higher concentration of monomethylamine or of 1 per cent. or 2 per cent. of the other amines produces a less satisfactory fibre structure.

The fibre structure of dry-salted goat skins is affected by the addition of sodium sulphide or methylamines to the lime liquor.

The addition of 0.1 per cent. monomethylamine to the lime liquor gives a limed pelt and chrome crust skin of the best fibre structure. The addition of 0.2 per cent. sodium sulphide also produces a skin of good fibre structure. Di- and trimethylamine in concentrations of 0.05 and 0.1 per cent. cause marked plumping of the fibres in the limed pelt and consequent disorganisation of the weave in the crust skin.

With both sheep and goat skins the use of methylamines in either the fellmongering paste or lime liquor produces a smoother grain on the crust skin than does the use of sulphide.

The authors wish to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

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"RUN" IN GLOVING LEATHER.
PARTS II AND III.

By
J. H. BOWES.

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September and October, 1942.

"RUN" IN GLOVING LEATHER.

PART II.—THE RELATIONSHIP BETWEEN "RUN" AND CERTAIN PHYSICAL PROPERTIES.

By J. H. Bowes.

(Reprinted from the *J.I.S.L.T.C.*, 1942, **26**, pages 181 to 203).

In a previous investigation[1] a number of gloving leathers were examined microscopically and an attempt made to correlate various microscopical features with " run " as measured by feel.

The assessment of " run " merely by feel, however, is not an altogether satisfactory method and is subject to various limitations. Without a great deal of experience difficulty is encountered in comparing leathers at different times and standards may vary not only from individual to individual but also from day to day in any one individual. Methods of measuring " run " and of obtaining a numerical assessment of this quality have, therefore, been considered.

" Run " as applied to gloving leathers is rather difficult to define, but a more clear idea of this quality may be reached by considering the methods normally employed in glove cutting. There are two main methods, block cutting and table cutting. In the first method the glove is cut out roughly to the shape of the hand. A leather with " run " is useless for this purpose since on inserting the hand, the glove will stretch and no longer fit the hand closely.

For table cutting, however, a skin with " run " is essential. The cutter works his skin first to length and cuts off the length required for the glove, he then pulls the skin at right-angles and cuts off a width. From this piece of leather, technically known as the trank, the actual glove is punched out. The cutter takes his measurements for the size of the glove he is cutting and if the leather is springy the trank will contract after cutting and be too small for the punch. Thus for table cutting, a leather is required which is not only extensible but will maintain this extension when the tension is released, i.e., it must possess plasticity rather than elasticity.

" Run " is also of importance in the finished glove; in order to fit the hand perfectly the glove must ease to the shape of the hand, but when removed and pulled length-wise should assume its original shape. The degree of extensibility and plasticity in both directions is, therefore, of importance.

Consideration of the above factors indicates that to obtain a satisfactory numerical value for " run " it is desirable to measure the extensibility and the degree of maintenance of this extension in several directions.

In this investigation, a large number of different measurements have been made on the same series of gloving leathers as that used in the earlier microscopical investigation. The extension in various directions under increasing load was measured. The leathers were also tested and the disten-

sion and pressure required for bursting measured on the Diaphragm Tester of the British Boot, Shoe and Allied Trades' Research Association. By kind permission of Sir Robert Pickard a number of these leathers were sent to the Shirley Institute and subjected to tests similar to those used in the examination of textiles,* including:—

1. Measurement of thickness, specific volume and incompressibility.
2. Measurement of stiffness—determination of bending length and flexural rigidity on the flexometer.
3. Determination of elastic properties by means of the Bursting Tester.
4. Tensile tests—(a) Measurements of extensibility and permanent set after application and release of a dead weight load; (b) Measurements of changes in width of samples under a given load.

On considering the results obtained as a whole it was found possible to relate " run " to certain measurable characteristics so that methods of testing are now indicated which may serve to assess " run " on a quantitative basis.

I. Physical Properties—Methods of Determination and Consideration of Results.

(i) Preparation of Samples.

The thirteen leathers examined covered a wide range of types, from a light kid suitable for women's gloves to a heavy hair sheep leather more suitable for men's gloves. The leathers were examined qualitatively by feel and arranged in decreasing order of " run." The differences in the degree of run were in most cases fairly great and easy to distinguish by feel and it can be safely assumed that this order is essentially correct, although a few individual skins which were difficult to place exactly may be slightly out of order. Throughout this investigation, this qualitative technical assessment by feel has been taken as a standard with which the results obtained by various physical tests may be compared. The leathers have been considered from the point of view of table cutting, in which run is a desirable quality, and therefore leathers have been classified as having good, fair or poor " run " according as to whether the degree of " run " varies from high to low.

It was impossible to carry out physical tests on the whole series of leathers which had been examined microscopically in the previous investigation since in some cases the samples were too small to admit of this. Also, it was not possible to carry out all tests on samples cut from the best portions of the skin, but as far as possible, samples from each series of tests were cut from corresponding portions of each skin. The approximate positions of sampling for the various tests are shown in Fig. 1. In the smaller skins the samples were relatively nearer the neck portion.

* The author would like to express her thanks to Dr. Peirce, under whose supervision these tests were carried out, for his help and criticism.

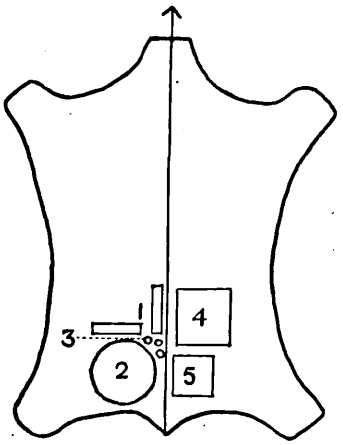

Fig. 1.

1. Samples for tensile tests carried out at the Shirley Institute.
2. Sample tested on Flexometer at the Shirley Institute.
3. Samples tested on the Diaphragm Tester in B.L.M.R.A. laboratories.
4. Samples tested for extension in the B.L.M.R.A. laboratories, using wide grips.
5. Samples tested for extension in the B.L.M.R.A. laboratories, using narrow grips.

The tests at the Shirley Institute were carried out under standard conditions of temperature and humidity, viz., 65 per cent. relative humidity and 65-70°F. The samples were conditioned prior to testing.

The extensibility tests in these laboratories were carried out at 65°F. and 60 per cent. R.H., while the tests on the Diaphragm Tester were carried out at 75°F. and 76 per cent. R.H.

(ii) Thickness, Specific Volume and Incompressibility.

The thickness of each sample was measured in three places in each of the sectors of the samples used for the stiffness determinations under pressures of 0, 1, 2, 5 and 10 lbs. per square inch. The diameter of the presser foot was three-eighths of an inch. The mean thickness in mm. of each sample at a pressure of 1 lb. per square inch is given in Table I. In every case the parts of the sample nearest to the backbone were the thickest.

From the thickness at 1 and 2 lbs. per square inch the compression modulus h in kilograms per square cm. was calculated from the equation $h = d \frac{\Delta P}{\Delta d}$ where d is the thickness at a pressure of 1 pound per square inch and Δd the change in thickness due to a change ΔP of pressure. The overall specific volume, i.e., the volume occupied by 1 gram of the leather, was also calculated. The values for the compression modulus and specific volume are also given in Table I.

There is a tendency for the thickness to increase as " run " decreases. This is probably due to the fact that the leathers with less " run " have come from older and larger animals whose skins are inclined to be thicker and less stretchy.

There is an indication that " run " may be associated with specific

volume, since values of about 3 for specific volume were found with leathers having good " run " and values of about 2 with leathers showing poor " run." The incompressibility generally increases as the " run " decreases.

TABLE I.
Some Physical Properties of the Leathers.

Leather No.	Weight mg. per sq. cm.	Thickness, mm. at pressure of 1 lb. per sq. inch	Specific volume	Incompressibility, kg. per sq. inch
1	16·3	0·46	2·81	0·84
2	16·0	0·51	3·21	0·98
3	19·3	0·54	2·80	1·17
5	26·2	0·83	3·15	1·07
6	28·0	0·72	2·58	1·29
9	33·1	0·99	2·99	1·33
12	76·4	1·84	2·41	1·21
16	49·3	0·98	1·98	1·13
17	50·3	1·03	2·05	1·80

(iii) Stiffness as measured by the Flexometer.

The stiffness of each leather was determined on the flexometer by the method described by Peirce[2] for textiles. This instrument and its application to the measurement of the stiffness of leather has been discussed by Conabere.[3].

The tests were carried out on circular samples 14 cm. in diameter, cut from the skin about 1 inch away from the backbone and 2 inches away from the tail edge. The discs were marked out into twelve equal sectors as shown in Fig. 2, the sector lying nearest the head being distinguished by an arrow.

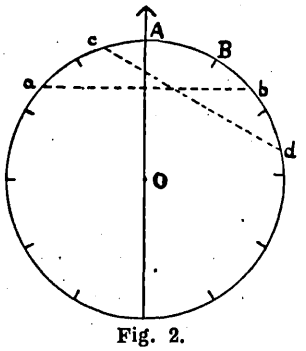

Fig. 2.

Diagram illustrating the measurement of the bending length in different directions.

The bending lengths in the twelve directions round the sample were then determined. The sample was first arranged on the flexometer so that the leather was bent along the line *ab* and the bending length in the direction *OA* calculated from the results obtained with three different lengths overhanging. The sample was then arranged so that the leather was bent along the line *cd* and the bending length in the direction *OB* determined. This was repeated in each of the directions round the circle. It must, therefore, be realised that the bending length and flexural rigidity in any given direction

are the values obtained when the leather is bent along a line perpendicular to this direction.

Suitable lengths were chosen throughout to give a range of bending from 20° to 50°. Measurements were made both with flesh and with grain surfaces uppermost. From the mean of the three values of the bending length c, the stiffness or flexural rigidity G was calculated from the equation $G = wc^3$, where w is the weight of the sample in mg. per sq. cm. In calculating G a correction was made for the thickness of the overhanging part of the leather. It was assumed that the weight per unit area of the overhanging part was proportional to its thickness, then

$$G = \frac{wd_1}{d} c^3$$

where d is the average thickness of the whole disc and d_1 the average thickness of the overhanging part of the leather.

The mean values for c and G are given in Table II. The bending length c gives a measure of the bending of the unsupported part of the leather when held in the hand. If c is small the projecting part will fall more steeply than if it is large, and gloves made from such leather will be more limp than those made from leathers with greater values for c. The flexural rigidity G measures the actual forces produced in bending the leather, leathers with large values of G will offer greater resistance to bending and so feel stiffer to the fingers than leathers with small values of G. Both the bending length and flexural rigidity increase as " run " decreases. The correlation with run is closer in the case of flexural rigidity, the values vary over a wide range and the differences between the leathers are more marked than in the case of the bending length.

TABLE II.
Bending Length and Flexural Rigidity.

Leather No.	1	2	3	5	6	7
Bending length in cm.	1·66	1·60	2·18	2·19	2·11	2·03
Flexural rigidity in mg. cm.	75·7	70·2	209	292	270	279

Leather No.	9	12	13	14	16	17	18
Bending length in cm.	2·02	3·60	2·24	2·41	2·35	2·65	2·68
Flexural rigidity in mg. cm.	270	3510	404	514	660	950	950

Leather No. 12 falls well out of order. This leather, although being fairly extensible was definitely rubbery and felt different from the other leather, and for this reason may not have been assessed accurately by feel in the first place.

The values for the flexural rigidity in different directions in each leather have been plotted as polar diagrams in which the radii are proportional to

the values of G in the corresponding directions. For example, the value of G obtained when the leather was bent along the line *ab* (see Fig. 2) is given on the vertical axis.

These polar diagrams show some degree of symmetry. This is understandable as opposite sectors were bent across the same direction. Flexural rigidity was in most cases greatest in the direction of the backbone, i.e., when the leathers were bent perpendicular to the backbone, although the actual direction of maximum flexural rigidity was often displaced to one side or the other. In general, if the flexural rigidity in one direction was large then that in the other directions was also fairly large. This probably explains why the average values for the flexural rigidity are found to show a relationship to " run."

The polar diagrams for Leathers No. 2, 6, 14 and 16 are reproduced in Fig. 3. That of Leather No. 2 is typical of leathers having good " run " and showing a directional variation. Leather No. 6 has less run and the difference in the flexural rigidity in different directions is less marked. Leathers No. 14 and 16 are typical of leathers having rather poor " run." The directions of maximum flexural rigidity are different in these two leathers.

It was observed that the flexural rigidity, measured with the flesh side of the leather uppermost is generally greater than that measured with the grain side uppermost.

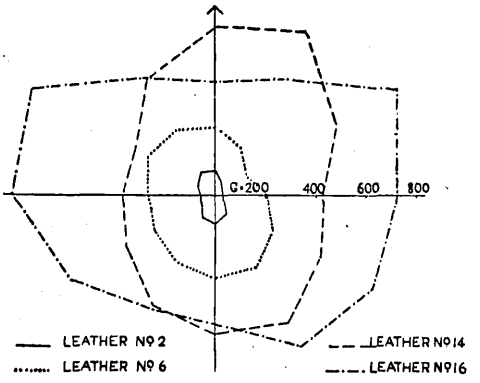

Fig. 3.
Polar diagrams showing variations of flexural rigidity in different directions for Leathers No. 2, 6, 14 and 16.

(iv) Elastic Properties as measured by the Bursting Tester.

These tests were carried out on the circular discs which had been used for the stiffness measurements. The samples were clamped under an annular foot 4 inches in internal diameter and distended by admitting water under pressure to the underside. A thin rubber diaphragm was used as a boundary between the water and the leather and corrections were applied to the results to compensate for the pressure necessary to distend the rubber. The percentage extension of the sample was calculated from the vertical rise of its centre assuming that it formed a spherical cap. The results of the tests are given in Table III.

TABLE III.

Leather No.	1	2	3	5	6	9	12	16	17
% Extension at 2 lb./sq. in. for ½ minute	10·0	11·0	7·8	7·8	8·8	11·2	14·2	9·4	8·5
% Extension at 2 lb./sq. in. for 15 minutes	10·6	11·4	8·2	8·3	9·3	11·9	14·5	9·8	8·8
% Extension at 2 lb./sq. in. after release	5·5	6·0	3·0	4·3	5·5	5·0	8·0	5·0	4·3
% Extension at 4·8 lb./sq. in. for ½ minute	12·0	15·2	10·5	10·2	11·4	13·5	19·0	11·5	10·1
% Extension at 4·8 lb./sq. in. for 15 minutes	12·6	16·0	10·9	10·9	11·8	14·1	19·5	12·1	10·4
% Extension at 4·8 lb./sq. in. after release	7·5	6·5	4·0	4·8	5·5	6·0	8·0	5·4	5·0
% Extension at burst	22·6	25·7	28·5	30·0	31·5	39·0	43·0	27·0	25·5
Bursting pressure, lb./sq. in.	24·5	13·5	38·0	38·0	51·1	81·1	68·9	44·0	73·8

The variation from leather to leather is in most cases comparatively small and does not vary directly with run, although there is some tendency for extensibility to decrease with decreasing " run." The effect of local weaknesses in the leather is probably too great to allow any close correlation to be observed. It was observed that in the burst specimens the direction of break lay along the backbone of the skin in many cases.

(v) Extensibility and Tensile Strength.

These tests were carried out on 1-inch wide strips cut both perpendicular and parallel to the backbone. The strips were mounted between grips 4 inches apart, a low tension (0.62 lbs.) was applied and the length measured. The strips were then loaded with a dead weight of 3 lbs. and the extension measured after half a minute, 5 and 15 minutes. The load was removed and the permanent set after 15 minutes was measured. Finally, the strips were broken in a tensile testing machine and the breaking load and extension recorded. The changes in width of the strips whilst under tension were measured, and found to be practically unaffected by time of loading. Only the results of the measurements attained by maintaining the load for 15 minutes are given in Table IV. The values obtained after half a minute and 5 minutes are omitted since they were of the same order but slightly less than the values obtained after 15 minutes.

It would be expected that the mean values for the percentage extension and percentage permanent set would be closely correlated to the degree of run of the samples as measured directly by feel. This, however, was not found to be so. The method of measuring the extensibility has obviously an influence on the results obtained and this question is discussed further when the values obtained for the extension in these laboratories is considered.

The decrease in width of the strips when loaded by a dead weight show a

TABLE IV.

Results of Tensile Tests.

Leather No.	1	2	3	5	6	9	12	16	17
TENSION OF 3 lb. FOR 15 MINUTES									
% Extension parallel to backbone	33	23	44	38	14	15	22	40	15
% Extension perpendicular to backbone	12	16	25	26	38	34	21	18	13
% Extension, mean value	22·5	24·5	34·5	62·0	26·0	24·5	21·5	29·0	24·0
% Permanent set parallel to backbone	31	21	39	33	12	12	19	36	14
% Permanent set perpendicular to backbone	11	12	22	24	34	21	16	14	11
% Permanent set, mean value	21·0	16·5	30·5	28·5	23·0	16·5	17·5	25·0	12·5
% Decrease in width, mean value	53	42	43	33	29	25	19	20	9
Ratio length/width, mean value	14·5	11·4	12·6	12·2	9·0	7·8	6·7	7·8	5·6
% Width decrease corresponding to 25% length decrease	58	49	32	17	30	31	24	19	17
Mean breaking load, lb./sq. in.	9·2	14·0	15·5	17·7	18·5	19·5	12·4	9·7	20·1
% Extension at break, mean value	48	56	72	64	67	63	74	65	44

definite relationship with " run." It seems very likely that such a measurement of change of shape will be closely related to the quality of " run " desired by the glove manufacturer.

Width change can be expressed in several ways, e.g.:—

1. Percentage decrease in width for a given load (3 lbs. in these tests).
2. Ratio of length to width of sample after application of the tension, the original values of the length and width being reckoned as unity.
3. Percentage decrease in width based on a constant extension, say, 25 per cent.

The figures obtained for the leathers by these three methods are shown in Table IV. By the first two methods the leathers are arranged in order of " run," the greatest decrease in width being found with samples having the best " run." Only the order of Leathers 2 and 3, and 12 and 16, requires changing in order to make the series coincide with the qualitative technical assessment. By the third method, Leather No. 5 falls well out of place and the whole range of variation is not so great as by the other methods.

In these tests, a weight of 3 lbs. was applied to a strip 1 inch wide for half a minute, 5 or 15 minutes, but the exact weight and time used are not important provided a specific procedure is adopted. A weight proportional to the weight of the leather might provide data more comparable over a wide range of materials, but for gloving leathers, one fixed weight seems to correspond better to the technical requirements.

Further measurements of extensibility were carried out in these laboratories using two widths of grip for holding the leather. In the first method samples approximately 10 cms. square were used. Toggle grips about 2 cms. wide were used to hold the leather and were placed 9 cm. apart. The upper toggle was fixed to an upright support bearing a vertical scale calibrated in cm. and a rod with a platform on which slotted weights could be piled was attached to the lower toggle. Weights were added gradually and the extension noted at the following tensions: 0.5, 0.75, 1, 2, 3, 4 and 5 kg. The weights were then removed one by one and the extensions again recorded. The extension of the leathers was measured parallel and perpendicular to the backbone and also in diagonal directions at 45° to the backbone. A preliminary stretching parallel to the backbone was given before any readings were taken in order that the maximum change in length in each direction should be measured. Graphs were drawn giving the curves for extension and recoil in each case. The percentage extension and the percentage permanent set were calculated for each leather and the results are given in Table V. The percentage permanent set is the percentage extension remaining after the leather has been released from tension and is thus a measure of the plasticity of the leather. In Fig. 4 are shown two typical curves, one for a leather having good " run " (Leather No. 2) and the other for a leather having poor " run " (Leather No. 17).

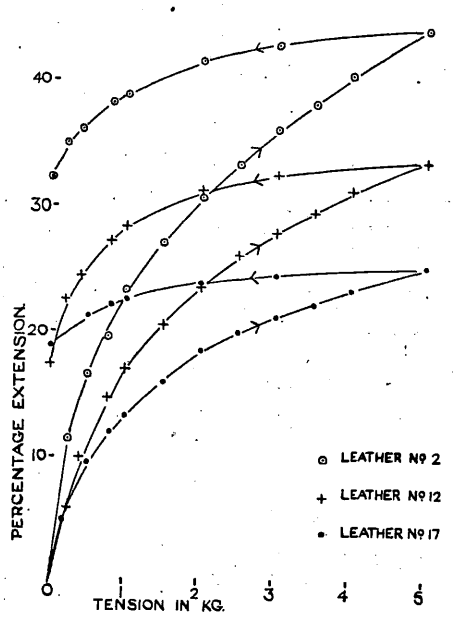

Fig. 4.

Graphs showing the curves for extension and release of leathers under given loads.

TABLE V.

Extensibility of Leathers in Different Directions.

Leather No.	1	2	3	5	6	7
% Extension at 5 Kg. Tension						
Parallel to backbone	43·4	38·5	41·4	40·6	26·1	28·6
Perpendicular to backbone	51·4	55·1	42·3	29·4	37·7	28·0
45° to backbone	44·4	36·6	51·8	34·9	37·2	25·7
315° to backbone	52·7	46·3	29·2	40·9	34·9	35·2
Mean value	48·0	44·3	44·1	36·6	34·0	33·6
% Permanent Set after Tension Released						
Parallel to backbone	34·3	28·0	33·1	30·3	16·3	22·0
Perpendicular to backbone	—	40·9	31·4	20·9	28·0	28·6
45° to backbone	33·7	26·0	37·8	26·0	28·6	17·7
315° to backbone	—	33·7	20·6	31·4	26·0	26·9
Mean value	—	32·3	30·9	27·2	24·9	23·7

Leather No.	9	12	13	14	16	17	18
% Extension at 5 Kg. Tension							
Parallel to backbone	42·9	32·0	33·4	30·8	28·6	23·4	21·7
Perpendicular to backbone	33·7	36·8	29·4	31·4	28·6	28·0	26·3
45° to backbone	28·0	29·1	27·2	26·3	25·7	24·9	24·3
315° to backbone	45·2	37·8	36·9	31·2	27·7	24·9	24·3
Mean value	34·1	33·7	31·7	30·0	27·7	25·4	24·3
% Permanent Set after Tension Released							
Parallel to backbone	30·0	17·1	25·7	21·1	18·6	17·1	13·1
Perpendicular to backbone	22·9	17·7	21·4	22·0	20·0	20·6	16·3
45° to backbone	18·3	14·3	20·6	17·4	15·4	17·7	14·9
315° to backbone	31·7	20·6	27·4	20·6	16·9	19·7	14·3
Mean value	25·7	17·4	23·7	20·3	17·7	18·9	14·6

The curve for Leather No. 12, which is also given, shows that this leather, although fairly extensible, tends to recoil to a great extent, so that the percentage permanent set for this leather is about the same as that of Leather No. 17 which was far less extensible.

The mean values for the percentage extension and percentage permanent set show a definite correlation with " run." If the leathers are arranged in order of decreasing values of extensibility, they fall approximately into the same order as that obtained for " run," only Leathers No. 9 and No. 7 having to be reversed. The leathers also fall into a similar series if the percentage permanent set is considered. In this case Leathers No. 7 and 9, and No. 16 and 17 are reversed and Leather No. 12 falls badly out of place, due no doubt to its rubbery quality already mentioned.

This method of measuring the extension using the small grips appears, therefore, to give results which may be used for the evaluation of " run." It was observed, however, that in using narrow grips of this type a waist

effect was obtained when the leathers were stretched and it was thought that this might lead to some error. The measurements were, therefore, repeated using larger samples and grips 10 cms. in width. These grips were made from three Meccano strips fixed together by three screws.

The values obtained for the percentage extension by this method varied over a smaller range. The maximum range of variation was 11.7 to 29.6 and the majority of the values were below 20. The mean values for each leather varied between 14 and 22 and appeared to have no connection with " run " as measured by feel. Consideration of the figures obtained for the percentage permanent set also appear to bear no relationship to " run."

A possible explanation of the discrepancy in the results obtained by these two methods can be obtained by consideration of the relationship between the width of the grip and the length of the leather subjected to extension. In the first method the length is more than four times the width of the grip, whilst in the second method the length and the width were the same. In measuring " run " by feel it is usual to stretch the skin first in one direction and then in the other. The hands are equivalent to the grips used in these experiments and the length of the skin equivalent to the length of the strip. It will be seen, therefore, that the first method, in which the grips are small, approximates more nearly to the conditions occurring in practice and for this reason gives results which can be correlated with the qualitative technical assessment of " run."

Consideration of the values for the percentage extension and percentage permanent set in different directions as determined by the first method, shows that in most leathers there is a direction of maximum and minimum extensibility. In some leathers the difference between the maximum and minimum values is more marked than in others, and in general, it is found that leathers having good " run " show a greater variation of extensibility in the different directions than do the leathers with less " run." In Fig. 5 are shown polar diagrams giving the percentage extension in different directions of three leathers, one having good " run " (Leather No. 2), another fair (Leather No. 9), and the third rather poor " run " (Leather No. 18).

In most cases the extensibility was greater when the leathers were stretched perpendicular to the backbone than when stretched in the direction of the backbone. The direction of maximum extensibility, however, does not necessarily lie either parallel or perpendicular to the backbone but may be in one of the diagonal directions. This suggests that, although in the original skin the directions of maximum and minimum extensibility are related to the direction of the backbone, these directions are modified by the finishing processes such as crushing and staking and may lie in any direction in the finished leather.

The effect of staking and straining while drying on the direction of maximum and minimum extensibility and flexural rigidity is further considered in Part III.

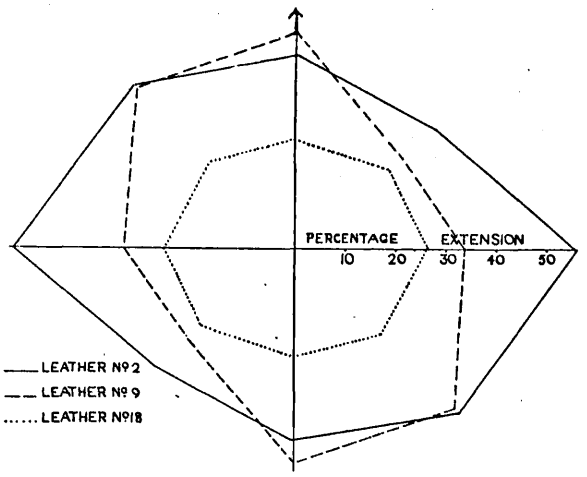

Fig. 5.

Polar diagrams showing variations in the percentage extension in different directions for Leathers No. 2, 9 and 18.

(vi) Distensibility and Bursting Pressure as measured by the Diaphragm Tester.

The leathers were tested for distension, crack and burst on the Diaphragm Tester. This instrument has been described in detail by Bradley[4] and McKay[5] and a short description of the instrument is also given by Conabere.[6]

In this investigation three duplicate samples were taken from each skin. The thickness of each was measured and the distensions at pressures of 1, 2, 3, etc. kg., up to the bursting pressure, were observed. Thickness, distension at pressure of 5 kg. and at the pressure where the leather cracked, and the pressures required to crack and to burst the leather, are recorded in Table VI, each figure being an average of three determinations.

The distension at 5 kg. does not vary greatly from leather to leather. There is a tendency for those leathers having good " run " to show greater distension than leathers having poor " run," but the distension does not differentiate further between the different degrees of " run." If the distension at 5 kg. is plotted against the thickness of the sample, there is found to be a relationship between the two properties. The decrease in distensibility is therefore probably due to the increasing thickness which accompanies decreasing " run " rather than to any direct relationship with " run."

The pressures at crack and burst show no direct correlation with " run." These values are a measure of the strength of the grain and corium rather than of the degree of " run " of the leather.

II. Consideration of the Physical Properties of the Leathers in Relation to " Run."

Consideration of the results as a whole indicates that many of the physical properties measured show some tendency to vary with " run " as assessed qualitatively by feel.

TABLE VI.

Distensibility and Bursting Pressure.

Leather No.	Thickness, mm.	Distension (mm.) at 5 kg. pressure	Distension (mm.) at crack	Pressure (kg.) at crack	Pressure (kg.) at burst
1	0·24	6·8	7·9	8	9
2	0·29	7·7	7·4	5	16
3	0·25	7·2	8·3	6	14
5	0·48	6·0	7·6	11	18
6	0·52	6·5	8·5	18	26
7	0·69	7·0	8·0	9	14
9	0·51	5·9	8·0	23	35
13	0·68	5·2	7·5	39	43
14	0·53	6·3	7·0	10	13
16	0·58	5·4	6·1	8	19
17	0·67	5·2	6·7	24	25
18	0·89	4·9	7·2	34	35

In many cases, however, the variations from one end of the series to the other are small and irregular and sufficient only to distinguish leathers of good " run " from those of poor " run." For instance, in the case of overall specific volume, it may be said that leathers having values of 2.8 or greater have good " run," whereas those having values of about 2 or less have poor " run," but it is not possible to differentiate further. Similarly, with the property of incompressibility, values of 1 or less can be associated with leathers having good " run," while leathers giving values greater than 1.5 may be assumed to have poor " run." The results obtained on the Bursting Tester and Diaphragm Tester give similar results, but the tendency to vary with " run " is rather less.

The variation in the mean values obtained for percentage extensibility according to the methods employed have already been discussed. The method adopted at the Shirley Institute and the method employed in this laboratory using large 10 cm. grips do not give results which can be related to " run." The values obtained using small grips, however, show a relationship to " run." Other properties which show a relationship with " run " are flexural rigidity as measured on the flexometer, and the change in width of strips on the application of tension. The latter may be expressed as percentage of decrease in width caused by a given weight for a given time or by the ratio of length to width when a given tension is applied, the original length and width being taken as unity. The mean values obtained in the above tests have been plotted against the number of the leathers arranged in order of

" run " (see Fig. 6). It will be seen that all the curves fall with decreasing " run " and follow the same general shape. Leather No. 12 has been excluded since it fell out of place in most cases owing to its peculiarly rubber-like properties.

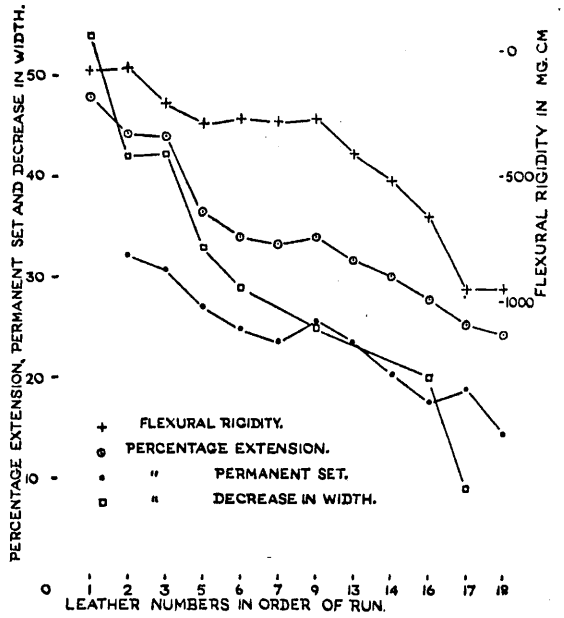

Fig. 6.

Graph showing the relationship between " run " and certain physical properties of the leathers.

It was stated earlier that in evaluating " run " from the glove manufacturer's viewpoint it was probably necessary to take into account the degree of " run " in different directions. In practice it has been found that, although there are directions of maximum and of minimum " run " (usually at right-angles to one another) as measured by the above methods, the degrees of " run " in the different directions are of the same order and therefore in most cases the mean values for the extension, flexural rigidity, etc., represent a fair estimate of the " run " of that skin in accordance with the qualitative technical assessment made by feel. Actually in evaluating " run " by assessment of the feel of the whole skin it is impossible not to take into account the degree of " run " in directions both parallel and perpendicular to the backbone. Great extensibility parallel to the backbone means correspondingly greater width-wise shrinkage and when the skin is stretched perpendicular to the backbone, the greater this width-wise shrinkage has been, the greater will appear the extension in this direction.

It may be assumed, therefore, that the mean values for these directional properties can be used to evaluate " run " on a numerical basis and an attempt can be made to give certain limiting values for varying degrees of " run " as follows:—

Flexural Rigidity.

Range of Values.	Assessment of "Run."	Leathers in each Group.
Less than 100 ...	Very good	1, 2.
100—200	Good	3.
200—400	Fair	5, 6, 7, 9, 13.
400—800	Rather poor ...	14, 16.
Over 800	Poor	12, 17, 18.

Percentage Extension at 5 Kg. Tension.

Range of Values.	Assessment of "Run."	Leathers in each Group.
Over 40	Very good	1, 2, 3.
35—40	Good	5.
30—35	Fair	6, 7, 9, 12, 13.
25—30	Rather poor ...	14, 16, 17.
Less than 25 ...	Poor	18.

Percentage Permanent Set after Application of 5 Kg. Tension.

Range of Values.	Assessment of "Run."	Leathers in each Group.
Over 30	Very good	2, 3.
25—30	Good	5.
20—25	Fair	6, 7, 9, 13.
15—20	Rather poor ...	14, 16, 17.
Less than 20 ...	Poor	12, 18.

Percentage Decrease in Width for Load of 3 lbs.

Range of Values.	Assessment of "Run."	Leathers in each Group.
Over 60	Very good	1.
45—60	Good	2, 3, 5.
30—45	Fair	6, 9.
20—30	Rather poor ...	16.
Less than 20 ...	Poor	12, 17.

It will be noted that the leathers fall into more or less the same groups, independently of which of these methods is used for evaluating "run." Further measurements on other leathers may necessitate the modification of the limits suggested, but as a first attempt at giving a numerical value to the quality of "run" they appear to afford a reasonable basis for assessment.

For practical purposes it will probably be most convenient to evaluate "run," by determining the flexural rigidity, since the values obtained for the extensibility of the leathers appear to vary according to the conditions under which the determinations are carried out and difficulties are involved in deciding what shall be taken, as the initial unstretched length of the sample. This is, therefore, not a very suitable method of evaluating "run" unless the conditions of determination are very carefully standardised. The same disadvantage is probably applicable to the evaluation of run by measurement of decrease in width. The flexural rigidity can easily be determined under standard conditions and is therefore probably the most suitable method for evaluating "run."

III. The Relationship Between Extensibility, Flexural Rigidity and Fibre Structure.

Since it has been suggested that the flexural rigidity shall be taken as a measure of the " run " it is of interest to consider the relationship between this property and the extensibility in different directions. In Fig. 7, the values for the flexural rigidity G in four directions in each sample are plotted against the corresponding values for the percentage extension. As would be expected from the previous discussion, there is a definite relationship, high values for the extensibility being associated with low values for the flexural rigidity.

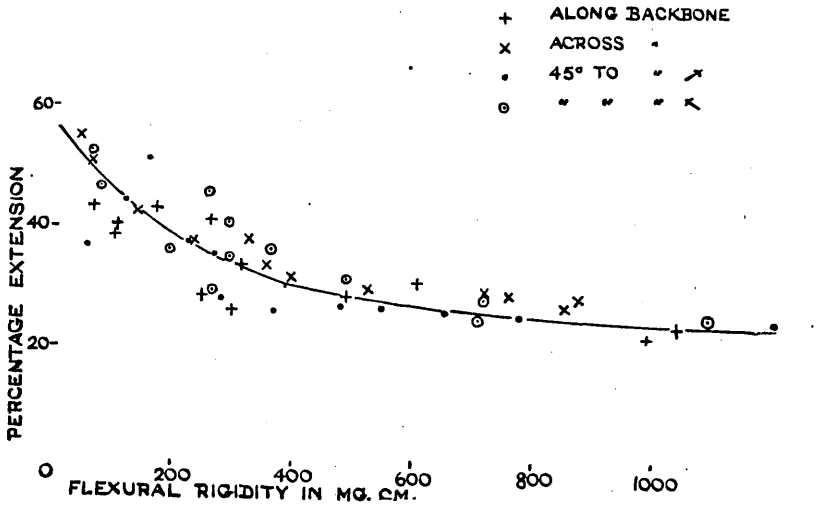

Fig. 7.

Graph showing the relationship between the Extensibility and Flexural Rigidity of the Leathers.

Comparison of the polar diagrams for the flexural rigidity and the percentage extension show that the leathers are most extensible in the direction in which the flexural rigidity is least. That is, if a leather has a small value for the flexural rigidity when bent about a line parallel to the backbone, then the extensibility when the leather is stretched perpendicular to the backbone is correspondingly large (see Figs. 2 and 4, Leather No. 2).

Conabere[7] has shown that in chrome calf upper leathers there is a relationship between the fibre structure and the stiffness in different directions. She has found that in sections cut perpendicular to the direction in which the flexural rigidity is least, the fibres are predominantly cut in a longitudinal direction, whilst in sections cut parallel to this direction, more cross-sections of fibres are visible, accompanied by a rather higher angle of weave. This indicates that flexural rigidity is least in a direction at right-angles to that in which the majority of the fibres are running.

The above conclusions arrived at for calf upper leathers do not necessarily apply to gloving leathers. These leathers are usually subjected to various

finishing processes, designed to increase the extensibility, which will tend to disturb the fibre weave of the original skin.

To test this, sections were cut parallel and perpendicular to the direction of minimum flexural rigidity in each sample. Microscopical examination showed that in every case where a direction of maximum and minimum stiffness could be distinguished numerically, a difference between the sections could be observed. There were many more cross-sections of fibres visible in the sections cut parallel to the direction of minimum flexural rigidity than in the sections cut perpendicular to this direction. In this latter direction, longer interweaving fibres could be seen and the angle of weave tended to be lower. Fig. 8 illustrates this. Photomicrograph A shows a section of Leather No. 2 cut perpendicular to the direction of minimum flexural rigidity, and B a section of the same leather cut at right-angles to this direction. Fig. 9, showing sections of Leather No. 18, illustrates this further. It therefore follows that flexural rigidity is least in a direction perpendicular to which the majority of the fibres are running. Since the directions of minimum flexural rigidity and greatest extensibility coincide, extensibility is also greatest in a direction perpendicular to that in which the most fibres run. This would suggest that extensibility is dependent on the separation of the fibres longitudinally as well as on the stretching of the fibres themselves. It would be expected that the extension produced by separation of the fibres longitudinally would be permanent, whereas the stretched fibres would tend to return to their original length.

The above discussion shows that both extensibility and flexural rigidity are dependent on the direction of the fibre weave in the leather. Assuming that the directional variations of the fibre structure are similar in all untreated skins, the fact that the direction of maximum and minimum extensibility and flexural rigidity are not always related in the same way to the position of the backbone, suggests that the finishing processes to which glove leathers are subjected are able to produce a certain amount of alteration of the fibre weave.

The effect of subjecting the skins to various strains during drying has been investigated and the results are reported on in Part III.

The author wishes to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

Summary.

1. The physical properties of a number of gloving leathers have been measured and the relationship of these properties to the quality of " run " as assessed by feel has been considered.

2. Certain properties such as flexural rigidity, extensibility and percentage decrease in width under a given load are found to be related to " run."

3. These properties have been used to evaluate " run " on a numerical basis and certain limiting values are given for varying degrees of " run."

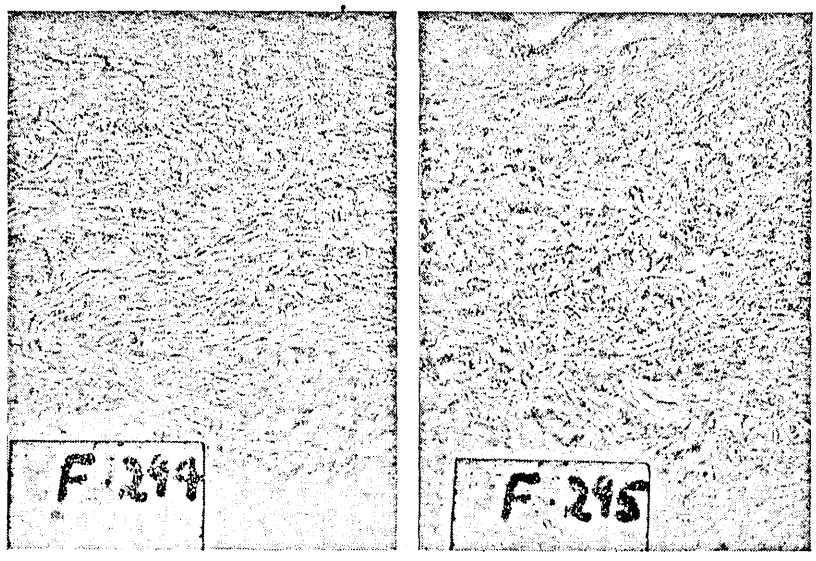

Fig. 8.

Photomicrographs showing the fibre structure of Leather No. 2.
A. Cut perpendicular to direction of minimum flexural rigidity.
B. Cut parallel to direction of minimum flexural rigidity.

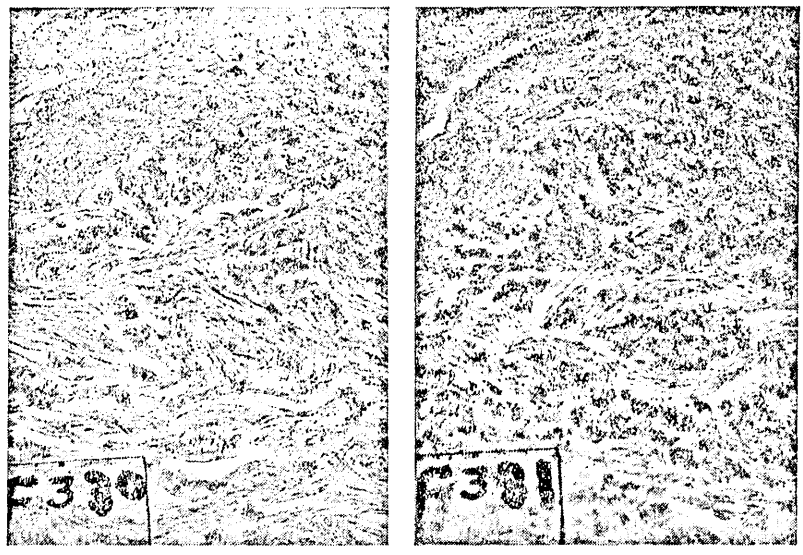

Fig. 9.

Photomicrographs showing the fibre structure of Leather No. 18.
A. Cut perpendicular to direction of minimum flexural rigidity.
B. Cut parallel to direction of minimum flexural rigidity.

4. It is suggested that flexural rigidity is the most practical method for evaluating " run." The leathers are most extensible in the direction in which the flexural rigidity is least.

5. Examination of the fibre structure of sections cut parallel and perpendicular to the direction of minimum flexural rigidity shows that stiffness in different directions can be associated with certain variations in the fibre weave. In sections cut perpendicular to the direction of minimum flexural rigidity, the fibres were predominantly cut in a longitudinal direction. The angle of weave was lower and more long interweaving fibres were visible than in sections cut parallel to the direction of minimum flexural rigidity. Therefore, the leather is least stiff and most extensible in a direction perpendicular to which the most fibres are running.

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(2) Peirce (1930), *J. Text. Inst.*, **21**, T377.
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(5) McKay (1936), *J.I.S.L.T.C.*, **20**, 9.
(6) Conabere (1941), *J.I.S.L.T.C.*, **25**, 281
(7) Conabere (1941), *J.I.S.L.T.C.*, **25**, 298.

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" RUN " IN GLOVING LEATHER.

PART III.—THE EFFECT OF STRAINING AND CRUSHING ON THE FLEXURAL RIGIDITY OF A NUMBER OF GLOVING LEATHERS.

By J. H. Bowes.

It was shown in Part II[1] that " run," as measured by extensibility and flexural rigidity, varies in different directions of the skin.

In general, it was found that the directions of maximum and minimum " run " lie approximately perpendicular and parallel to the backbone respectively, but in a few cases the directions were reversed. These variations in direction were considered to be due to the methods of processing such as crushing (or stocking), staking and straining to which the skins had been subjected. It was, therefore, decided to carry out experiments in order to determine the effect of crushing and of straining the leathers in different directions during drying.

The leathers used in this investigation were kindly supplied by Messrs. Dent Allcroft, who also carried out the straining and crushing processes.

Experimental Procedure.

The leathers examined had been treated as follows:—

Leather No. 1—Alum tanned, Spanish lamb skin—dyed fawn. No straining.

Leather No. 2—Alum tanned, kid skin—undyed. No straining.

Leather No. 3—Alum tanned, kid skin—undyed. Dried tacked out.

Leather No. 4—Alum tanned, kid skin—undyed. Strained along backbone.

Leather No. 5—Alum tanned, kid skin—undyed. Strained across backbone.

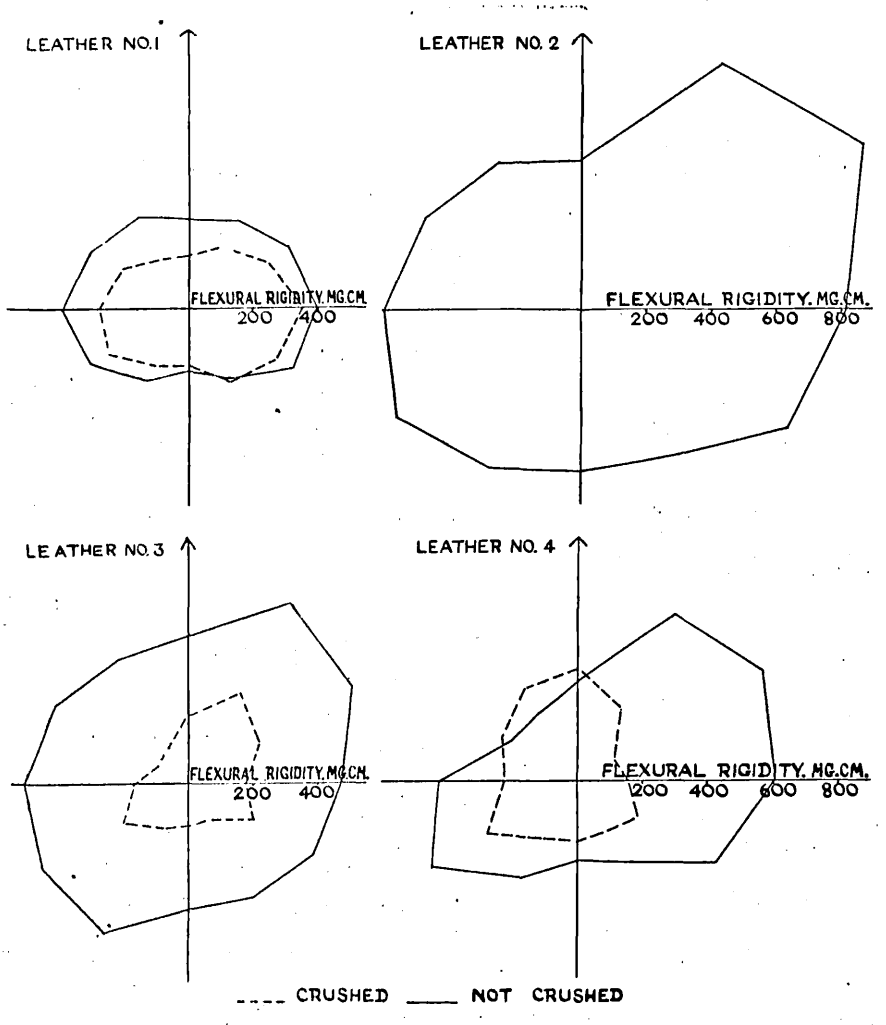

Fig. 1.

Polar diagrams giving the flexural rigidity of the leathers in different directions.

Leather No. 1—Alum tanned Spanish lambskin—not strained.

Leather No. 2—Alum tanned kid skin—not strained.

Leather No. 3—Alum tanned kid skin—strained in all directions.

Leather No. 4—Alum tanned kid skin—strained along backbone.

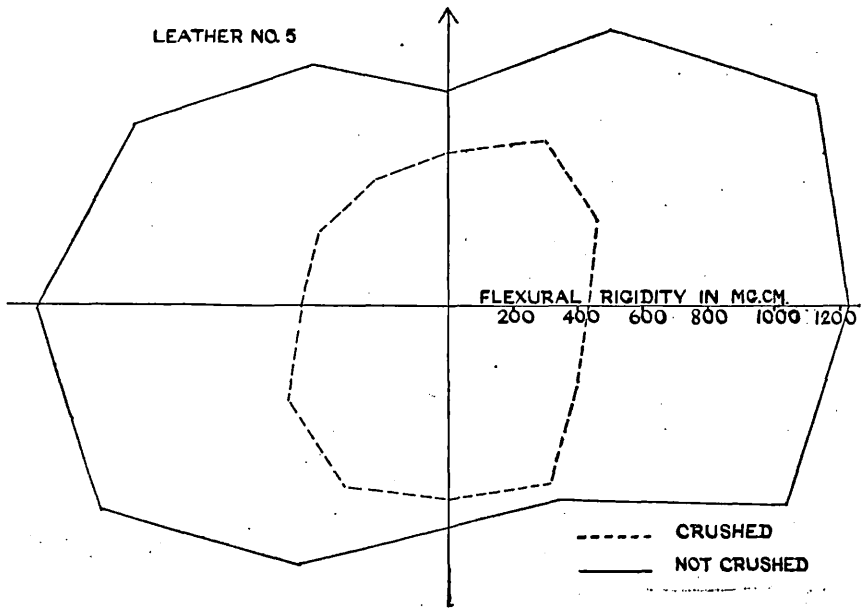

Fig. 2.

Leather No. 5—Alum tanned kid skin—strained across backbone. Polar diagram giving the flexural rigidity in different directions.

After straining the skins were divided down the backbone and one half crushed. Crushing consisted of a light stocking carried out on the dry leather with the addition of a little French chalk to lubricate the skins so that they turned easily. Circular samples were cut from the skins about 1 inch away from the backbone and 2 inches in from the tail. The flexural rigidity in twelve directions was determined for each sample as described in Part II. Polar diagrams of the leathers before and after crushing are given in Figs. 1 and 2.

Discussion of Results.

It will be noticed that there is a considerable variation in the flexural rigidity from sample to sample. These differences are probably due to differences in the original skins. It was not, therefore, possible to draw any conclusions with regard to the effect of the straining on the flexural rigidity of the skins as a whole, since unless a large number of samples are examined the individual variations from skin to skin cannot be eliminated. It is probably safe to say, however, that before crushing the Spanish lamb skin is more flexible than the kid skins, but is less affected by this process than are the other skins. Contrary to expectation straining in different directions had little obvious effect on directions of maximum and minimum flexural rigidity. Crushing reduced the flexural rigidity to a marked extent and minimised the variation in flexural rigidity in different directions.

In the kid skins, crushing also affected the direction of maximum stiffness; before crushing this lay between 60° to 90° to the backbone, while after crushing it was approximately parallel to the backbone. It is probable, therefore, that even if straining initially has some effect on the stiffness in different directions, this will disappear on crushing.

The author wishes to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

Reference.

(1) Bowes (1942), *J.I.S.L.T.C.*, **26**, 181, *ibid.* 189.

THE EFFECT OF STORAGE, IN THE SALTED AND IN THE PICKLED CONDITION, ON THE FIBRE STRUCTURE AND GREASE CONTENT OF SHEEPSKINS AND GOATSKINS.

By
MICHAEL P. BALFE, JOANE H. BOWES,
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October, 1940.

THE EFFECT OF STORAGE, IN THE SALTED AND IN THE PICKLED CONDITION, ON THE FIBRE STRUCTURE AND GREASE CONTENT OF SHEEPSKINS AND GOATSKINS.*

By Michael P. Balfe, Joane H. Bowes, R. Faraday Innes and Winnifred B. Pleass.

(Reprinted from the *J.I.S.L.T.C.*, 1940, **24**, pages 329 to 356).

Introduction.

This work was undertaken to obtain information about the natural grease of sheep- and goatskins and to determine what changes take place during the storage of such skins in the wool or hair or after depilation. The present report describes a study of the changes in the fibre structure, and in the distribution and composition of the grease, which take place during the storage of sheep- and goatskins in the pickled and in the salted conditions. Most of the recent work carried out has been confined to a study of the grease present in hides. Koppenhoeffer (1936) states that three types of grease, differing in chemical composition may be found in hides. These are the greases similar in composition to wool grease (lanolin), the triglyceride greases (the neutral fats), and the greases which contain compounds of fatty acids and phosphoric acid (phospholipins).

Koppenhoeffer and Highberger (1934) have found that grease of the wool grease type and of the phospholipin type are mainly present in the grain layer. The amount of phospholipin grease present in hides and skins is small.

Koppenhoeffer (1936) states that the corium of ox-hide contains about 0.5 per cent. of phospholipin (on the fresh skin weight), and according to Koppenhoeffer and Highberger (1934) the grain layer contains about twice as much phospholipin as the corium.

The triglyceride portion of the grease in hides varies in amount between wider limits than do the other two types of grease (Koppenhoeffer and Highberger, 1934). These workers find the grease present in the corium consists to a large extent of triglycerides and probably represents the fat contained in the fat cells of the corium. The amount of triglyceride grease varies from

* This paper is based on a report published in the Laboratory Reports of the British Leather Manufacturers' Research Association in 1937.

animal to animal, and it may be considered that variations in the grease content of skins is mainly due to variations in the amount of triglyceride grease.

The grease content of sheepskins (from domestic sheep) is higher than that of most other hides or skins. In different skins, and in different areas of the same skin, it may vary between 10 and 100 g. grease per 100 g. collagen. The grease content of goatskins, which is usually lower than that of sheepskins, may also vary within wide limits, as shown in Tables I and VI.

TABLE I
Grease Contents of Goatskins

Type of cure	Source of skin	g. Grease per 100 g. air-dry skin	% of total grease extracted by light petroleum*	— Analysis of grease — % Free fatty acid	% Unsaponifiable matter
Drysalted ...	Cape	15·4	93	15·6	3·7
	Cape†	57·0	—	5·8	0·3
	Cape	12·0	—	26·6	3·9
	Patna‡	3·8	71	42·1	—
	Patna‡	6·0	80	56·6	—
	Patna	13·0	83	30·4	—
	Patna	10·1	83	29·7	—
	Dacca	6·9	90	76·9	—
Sun-dried ...	Kampala ...	6·6	73	38·4	—
	Calcutta ...	4·9	82	36·8	—
	Cape	5·9	90	43·0	—
	Tangier ...	1·8	78	12·3	—
	Dacca	3·2	78	7·9	—
	Nigeria ...	5·0	—	24·2	—
	Nigeria ...	8·3	—	12·8	—
	Nigeria† ...	17·9	—	23·3	—
	Nigeria† ...	14·2	—	13·0	—

* 3 hours' extraction in Soxhlet apparatus
† Greasy skin
‡ Skin showing mould growth

Koppenhoeffer (1937) has also studied the changes taking place in the composition of the grease from steer hides after flaying, after storage in the salted condition and after liming. He has shown that hydrolysis of grease in steer hides commences within 24 hours of flaying and continues during storage in the salted condition, and that appreciable oxidation of the grease does not occur during storage. These observations are in harmony with those made in the present investigation. Koppenhoeffer also found that after storage of the salted hide for 6 months the phospholipins were completely decomposed, and that liming decomposes the phospholipins and neutralises free fatty acids, but does not affect the corium triglycerides.*

* Since the original publication of this report a number of papers by Koppenhoeffer and co-workers have appeared—
Koppenhoeffer, *J.A.L.C.A.*, 1937, **32**, 627 and 637;
Koppenhoeffer, *J.A.L.C.A.*, 1938, **33**, 79 and 203;
Koppenhoeffer, *J.A.L.C.A.*, 1939, **34**, 240 and 380.
These papers report work carried out on the lipids of hides and sheep and goatskins. Exhaustive analyses of the grease in these skins have been made and the effect of various processes such as curing, liming and bating on the composition of the grease has been determined.

A part of the grease in hides and skins cannot be extracted with grease solvents, but can only be isolated after hydrolysis of the collagen. See Fahrion (1910), Stiasny (1931), McLaughlin and Theis (1924, 1925 and 1926), Koppenhoeffer, 1936 and 1937).

The amount of this unextractable grease is not likely to exceed 1 per cent. of the air-dried weight of skin. It is, therefore, of minor importance from the standpoint of the present investigation, since the results obtained show that very wide variations may exist in the rate at which the grease can be removed, particularly from sheepskins, by light petroleum. These variations result in considerable differences in the amounts of grease which can be removed from the skin under different conditions.

Experimental Material and Methods.

(i) Preparation of the Experimental Pieces.

Both the sheep and goatskins used in this experiment were received within six hours of slaughtering and flaying, and were at once cut along the backbone. The right sides were cut into quarters which were pickled or salted at once. The left sides were sent to a fellmonger, who removed the wool or hair, using a paint which contained 6 g. crystalline sodium sulphide per 100 c.c. filtered solution, and returned the sides to the laboratory. The unhaired sides were limed for two days in 10 litres of liquor containing 4 per cent. of lime and 2 per cent. of gelatin (to simulate the action of the products of the hydrolysis of proteins which are present in old lime liquors), and then limed for 4 days in 10 litres of liquor containing 4 per cent. of lime, and no gelatin. The proportions of lime in the liquors are calculated as quicklime. After liming the sides were washed with water and then delimed during 3 hours in a 0.5 per cent. solution of ammonium chloride, which brought them to a pH value of approximately 7.5. They were then washed, drained and cut in half, the two quarter skins being at once pickled or salted.

The two shoulder quarters were each pickled in 4 litres of a liquor which contained 1 per cent. of sulphuric acid and 10 per cent. of salt. These concentrations have been shown to give the most satisfactory results in pickling, and pelts pickled in these conditions give aqueous extracts having pH values of 2.0 to 2.2 (Pleass, 1934). After 1 hour the liquors were poured off and replaced by 12 litres of liquor of the same composition. The quarters were left in the second liquors overnight, removed and drained. After use the second liquor contained 0.8 per cent. of sulphuric acid. A portion of the dewooled sheepskin was pickled in a liquor which contained a low concentration of acid, in order to allow the development of mould. This liquor originally contained 0.5 per cent. of sulphuric acid and 10 per cent. of salt, and after use it contained 0.3 per cent. of sulphuric acid. Pelts pickled in these conditions give aqueous extracts with pH values above 2.4 at the time of pickling, and as the storage of the pelts is prolonged mould growth develops which is accompanied by an increase in the pH value of the aqueous extract.

The butt quarters (one in the hair or wool, and one unhaired or dewooled) were salted on the flesh side with medium grain salt. This was removed

after 24 hours and a second application of salt was given and the pieces allowed to dry. Each application of salt was approximately equal in weight to the sample to which it was applied.

The pickled quarters were stored in stoppered jars in an incubator at 25°C. and the salted quarters, rolled up, with the adhering salt, were stored in the same incubator; during storage the salted quarters dried to varying extents. Immediately on receipt of the skins, immediately after liming and immediately after pickling or salting, samples were taken for microscopical and chemical examination. During storage, samples were taken from the pickled quarters after 6 weeks, 3 months, 8 months and 15 months, and from the salted quarters after 3 months and 8 months. The positions from which the samples were taken are shown in Fig. 1.

(ii) Microscopical Technique.

The samples for microscopical examination were hardened by immersion for one week in an aqueous solution of formaldehyde (4 per cent.) and sodium chloride (0.5 per cent.). Sections were then cut on the freezing microtome and mounted in Farrant's solution. Photomicrographs of these were taken at a magnification of 86 in order to have a record of the fibre structure of the corium of the samples. Sections were also stained for fat, using scarlet red in 70 per cent. alcohol. Photomicrographs of the stained sections, mounted in Farrant's solution, were taken at a magnification of 20 in order that the distribution of fat in both the grain and corium layers of the skin might be shown on the same photomicrograph.

The salted samples had become very dry after 8 months' storage and microscopical examination revealed little detail in the fibre structure. Some of the samples were therefore washed in water for a day prior to fixing in formalin for section cutting. After this procedure the sections were slightly more opened up but the improvement was not very marked.

(iii) Analytical Methods.

The wool or hair was closely clipped from the samples from the right sides, and all the samples were dried in a current of air at laboratory temperature until constant in weight, before analysis. The grease was then removed from the samples (which were not sub-divided) by two successive extractions of 3 hours duration, in a Soxhlet apparatus, with light petroleum (b.p. 40-60°). The greases removed in the two extractions were weighed separately. The degreased samples were hydrolysed by boiling for 3 hours with 2*N* alcoholic potassium hydroxide solution (5 c.c. per g. skin). The alcohol was removed by distillation, and the residue, dissolved in water, was acidified with hydrochloric acid and extracted with ethyl ether, which dissolves fatty acids and oxidised fatty acids. After removal of the ether by distillation, the residue of fatty acids was dried at 98°C. till constant in weight.

The consistency and colour of each specimen of grease was recorded. The free fatty acids, unsaponifiable matter and oxidised fatty acids in the combined greases from the two extractions with light petroleum were determined.

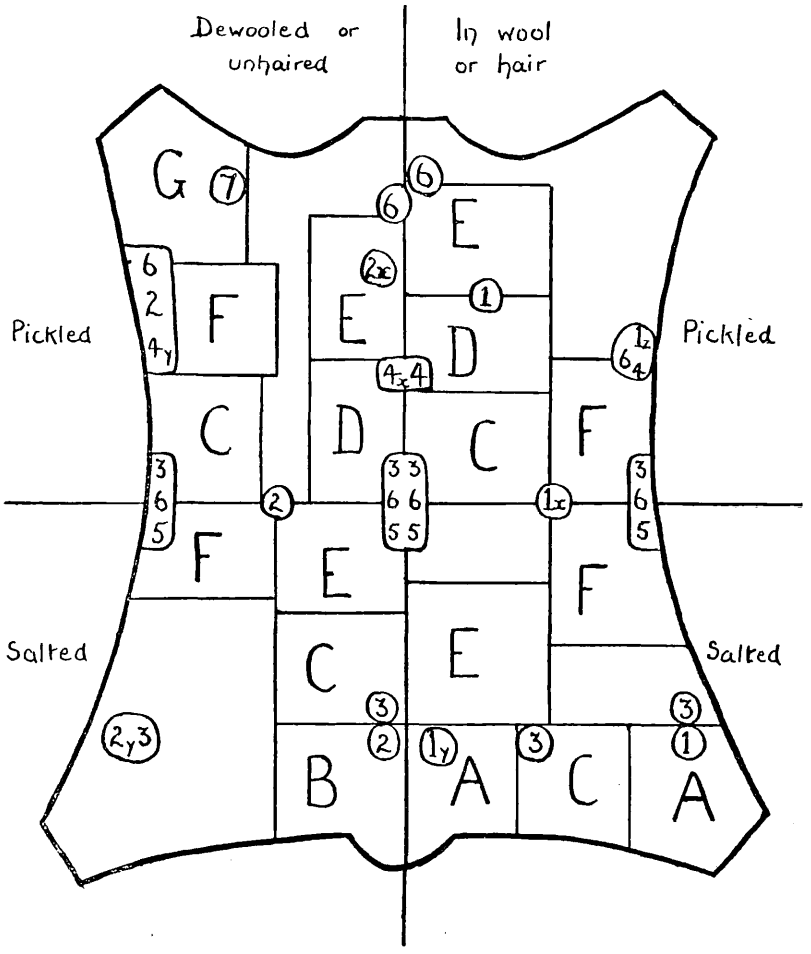

Fig. 1

Sampling Positions

	Samples for microscopical examination	Samples for analysis
Fresh skin	1, 1*x*, 1*y*, 1*z*	A
Limed skin	2, 2*x*, 2*y*	B
Immediately after salting or pickling ...	3	C
Stored for 6 weeks	4, 4*x*, 4*y*	D
Stored for 3 months	5	E
Stored for 8 months	6	F
Sheepskin pickled with deficiency of acid	7	G

The amounts of phospholipins in the greases was not determined, since owing to the small quantities in which they occur (Koppenhoeffer and Highberger, 1934, Koppenhoeffer, 1936), it appeared that they would be less important than the triglyceride or wool greases.

The amounts of grease available for analysis varied between 0.5 and 5 g. The three analyses were therefore made on one portion of grease. The free fatty acid contents were determined by titration in cold alcoholic solution with 0.1 *N* sodium hydroxide solution. After this titration 50 c.c. of *N* alcoholic potassium hydroxide per g. fat were added, and the solution refluxed for 3 hours to saponify the grease.

The oxidised fatty acids were determined by the usual method (Procter, 1919). The unsaponifiable matter was extracted with light petroleum and refluxed again with 2.5 c.c. *N* alcoholic potassium hydroxide for 4 hours, to ensure that no sterol esters remained. The unsaponifiable matter was again isolated from the alkaline solution, dried and weighed. The loss of weight on resaponification was in no case more than 5 per cent. of the original weight of unsaponified matter. The fatty acids derived from the greases were in some cases isolated from the aqueous layer and their melting points and equivalent weights were determined. The amounts of fatty acids obtained by hydrolysis of the degreased specimens were too small for further examination.

General Condition of Samples after Storage.

Table II records the extent to which mould growth occurred on the experimental pieces during storage.

TABLE II
Extent of Mould Growth on Samples during Storage

Duration of storage	SHEEPSKIN				
	In the wool		Dewooled		Pickled with deficiency of acid
	Pickled	Salted	Pickled	Salted	
6 weeks	−	+	−	−	++
3 months	−	++	−	−	++
8 months	−	++	−	−	++
15 months	++	++	−	+	++
26 months	−	++	−	+	

Duration of storage	GOATSKIN			
	In the hair		Unhaired	
	Pickled	Salted	Pickled	Salted
6 weeks	+	++	−	−
3 months	+	++	−	−
8 months	+	++	−	−
15 months	+	++	−	−
26 months	++	++	−	−

− No visible mould growth
\+ Slight mould growth
++ Heavy mould growth

Extensive mould growth and red heat was observed on the sheepskin salted in the wool after three months' storage. The goatskin salted in the hair also showed mould growth after three months. Only slight mould growth was observed on the depilated samples after 15 months' storage.

After 26 months the salted samples had become very much dried and the grain had been ruptured by crystallisation of the salt. Apart from this

all the samples were sound, although a few colonies of mould were observed on the samples which had been salted in the wool or hair.

In the case of the pickled samples no mould growth was observed on the depilated samples even after 26 months' storage. Mould growth developed on the woolskin after 15 months and on the goatskin in the hair after only 6 weeks.

The goatskin in the hair was still in fairly good condition after 26 months' storage, but the skin had become brownish yellow and the hair was loose. The sheepskin in the wool had deteriorated to a marked extent. It was very tender, the wool was loose and the sample had become very much blackened. The mould present after 15 months' storage was no longer visible.

Since mould growth is generally associated with an increase in pH value of the pickled pelt the pH values of the water extract from the degreased pickled pelts were determined (see Table III). The salt contents of the samples taken after 3 and 8 months' storage were also determined (Table IV).

TABLE III

pH Values of Aqueous Extracts from Pickled Samples After Storage for Eight and Twenty-Six Months

	pH value	
	8 months	26 months
Sheepskin in wool (wool clipped off before extraction of sample)	4·45	8·0
Dewooled sheepskin, normal pickle	2·18	2·0
Dewooled sheepskin, pickle deficient in acid	4·30	—
Goatskin in hair (hair clipped off before extraction of sample)	3·20	3·5
Unhaired goatskin, normal pickle	2·33	2·4

TABLE IV

Average Salt Contents of Samples

(g. NaCl per 100 g. air-dry degreased skin)

	In the hair		Unhaired	
	Pickled	Salted	Pickled	Salted
Sheepskin	24	33 ...	24	47
Goatskin	14	22 ...	19	48

The sheepskin pickled with deficiency of acid contained 36 g. salt per 100 g. air-dried degreased skin

The pH values of the aqueous extracts of the sheepskin in the wool and to a lesser extent of the goatskin in the hair, after storage for 8 months were considerably higher than was expected. Since the original pickling was carried out in liquors of standard composition and in each case to ensure thorough treatment the skins were immersed in two pickle liquors, it is unlikely that the pelt did not originally take up its full quota of acid. Therefore, it seems probable that during storage the acidity of these skins diminished, thus leaving an insufficient quantity of acid in the skin to prevent the growth of mould.

Samples were again examined after 26 months' storage. The pH values of the skins were determined roughly by means of indicators (see Table III).

In the case of the dewooled sheepskins there was no apparent loss of acid during further storage, but in the case of the sheepskin pickled in the wool a considerable amount of acid had been lost. This suggested that, with the skin in the wool, acid might have passed from the pelt to the wool. The pelt and wool were, therefore, examined by two methods in order to throw more light on this point. The pH values of the aqueous extracts of the pelt and wool were determined separately and Procter-Searle determinations carried out in order to estimate the amount of sulphuric acid originally present. During storage some of the sulphuric acid in the pelt may have been converted to ammonium sulphate. Procter-Searle determinations will return both sulphuric acid and ammonium sulphate and therefore gives a value for the amount of sulphuric acid originally present. These values and corresponding figures for a freshly pickled skin are given in Table V.

TABLE V

	Freshly pickled		Pickled for 2 years	
	Pelt	Wool	Pelt	Wool
pH of aqueous extract	2·02	2·02	8·00 8·41	7·86 8·02
Procter-Searle value, expressed as percentage H_2SO_4 on the drained weight ...	1·6	1·7	2·4 1·8	1·9 1·3

These results show that the acid is fairly evenly distributed between the pelt and wool of the freshly pickled skin and that in the skin pickled for 26 months the wool does not contain more ammonium sulphate (originally sulphuric acid) than does the pelt. A possible explanation might be that the interfibrillary proteins which are normally removed during dewooling and liming are hydrolysed more readily by the acid than the fibrous proteins. During acid hydrolysis of proteins the peptide links are ruptured, giving rise to polypeptides, amino acids and even ammonia. This will result in an increase in the number of basic groups available for combination with the sulphuric acid. After hydrolysis the sulphuric acid will therefore be present either as ammonium sulphate or as sulphates of other nitrogenous bases.

The samples which had been stored for 26 months were chrome tanned, fat liquored and dried out.

The chrome tanned pickled samples were full and pliable, the sheepskin samples being especially full. The samples were not easily torn except in the case of the sheepskin pickled in the wool.

The chrome tanned salted samples were also not easily torn. The sample of sheepskin in the wool was full and pliable, but that of the dewooled skin was flat though pliable and was a bad colour, being almost black with salt specks on the grain. The sample of goat skin in the hair was full but rather firm, while the unhaired sample was full and fairly pliable, but the grain again showed salt specks.

Microscopical Examination of Samples.

(i) SHEEPSKIN.

(*a*) *Fresh Skin.*

Examination of sections cut from five different parts of the fresh sheepskin showed that while the variations in fibre structure in different parts of the skin were not very great, towards the flank the angle of weave was rather lower and the fibres rather more loosely woven than near the backbone. A large number of fat cells were apparent. The sebaceous and sudoriferous glands were clearly shown, these were not visible after pickling, salting or liming.

(*b*) *In the Wool, Pickled.*

The fibre structure of the sheepskin in the wool, immediately after pickling, is shown in Fig. 2. The fibres were fuller and more regularly woven than in the fresh skin. After storing for three months there was little change in the fibre structure. After 8 months, the fibres were found to be very much opened up. This was probably due to the action of the pickle liquor, in weakening the reticular tissue round the fibres and fibrils.

The fibre structure of the sample which had been chrome tanned after 26 months' storage showed definite damage, though this is not so pronounced as would be expected from the macroscopical appearance of the pickled sample. The fibre weave of the corium was disorganised, the fibres appeared thin and stuck together and there was a looseness at the junction of the grain and corium.

(*c*) *In the Wool, Salted.*

The fibre structure of the skin after salting was not as clearly defined as that of the fresh or pickled skins (Fig. 3). This suggests that salting has caused a shrinkage of the fibres.

Comparison of all photomicrographs of salted skins with those of fresh or pickled skins showed that sections of the salted skin were often not clearly defined, probably due to partial dehydration of the fibres taking place in the salted condition. After the samples had been stored for 8 months, although they were washed in water prior to fixing in formalin and sectioning, the fibres had obviously become much dehydrated. The fibres were glassy in appearance with very little fibril structure apparent and were very horizontally woven (Fig. 4).

Extensive mould growth and red heat was observed on the specimen after 3 months' storage; microscopical examination showed that mould hyphæ and cocci were penetrating into the corium from the flesh side.

The fibre structure of the chrome tanned sample was definitely poor. The fibres were thin, little opened up, and the weave horizontal. There were many open spaces and definite indications of damage having occurred.

(*d*) *Dewooled, Pickled.*

After pickling the fibre structure was similar to that of the specimen pickled in the wool. As with the skin pickled in the wool, prolonged storage caused an increase in the splitting up of the fibres. The fibre structure of a sample which had been stored for 8 months is shown in Fig. 5. The translucent appearance of the section is due to a film of fat on the fibres.

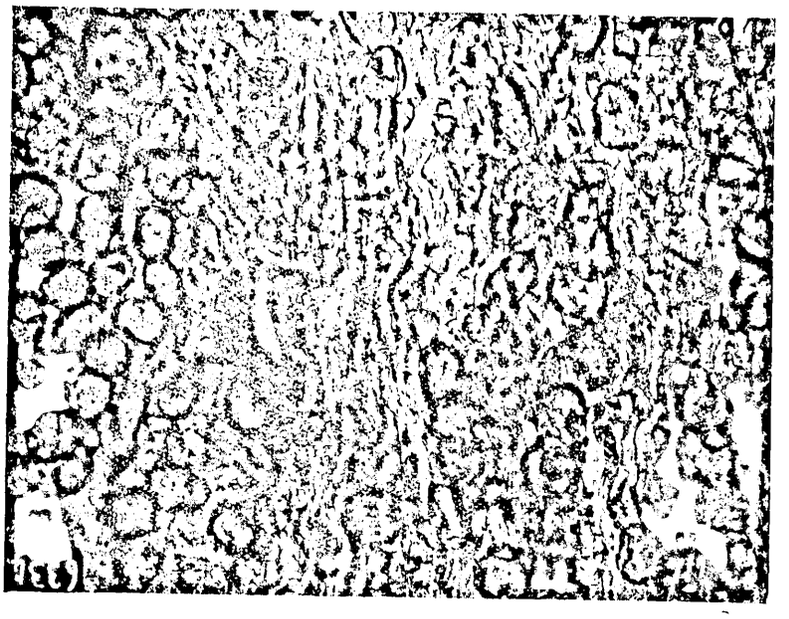

Fig. 3.
Freshly salted sheepskin in the wool. (× 86).

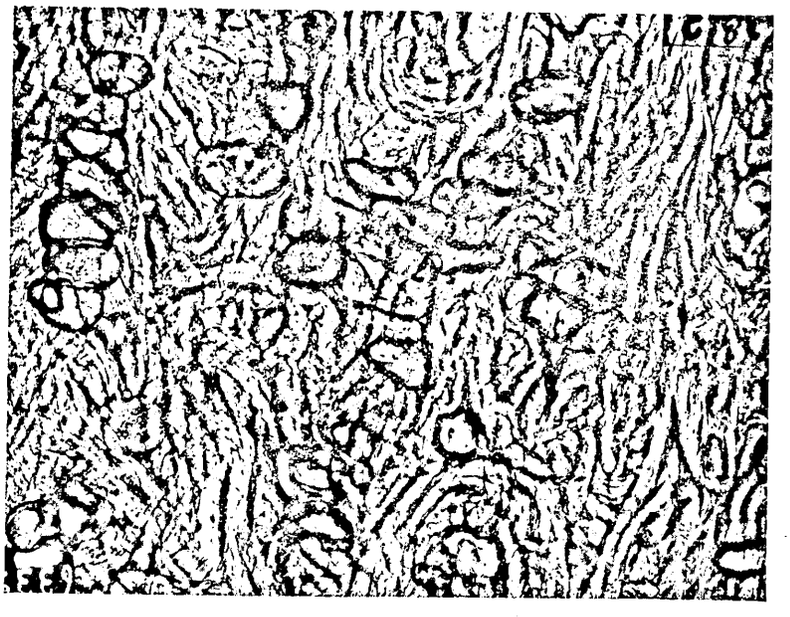

Fig. 2.
Freshly pickled sheepskin in the wool. (× 86).

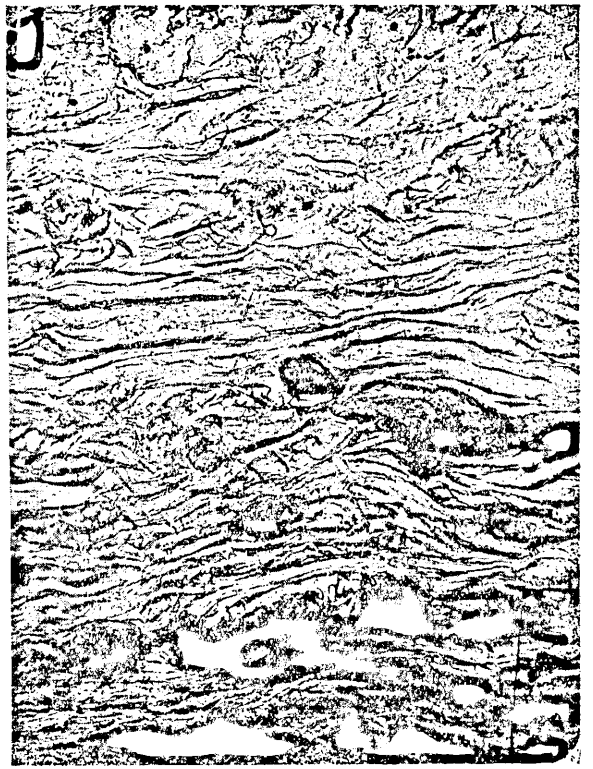

Fig. 4.
Salted sheepskin in the wool after storage for 8 months. (× 86).

Fig. 5.
Pickled fellmongered sheepskin after storage for 8 months. (× 86).

On storage the changes in the sample which had been pickled in a liquor containing a deficiency of acid were similar, generally, to those of the well pickled sample but the mould growth which occurred within a few weeks caused extensive damage to the fibre structure. The weave pattern was disorganised and loose and the fibres very much split up and separated.

The fibre structure of the chrome tanned sample was better than of the corresponding sample pickled in the wool. The fibres were less disorganised, but the angle of weave was again rather flat and open spaces were visible showing that some degradation had taken place.

(*c*) *Dewooled, Salted.*

The fibre structure of the freshly salted fellmongered skin was very similar to that of the skin in the wool immediately after salting. During storage the salted fellmongered skin became so dehydrated that many of the fibres became glassy in appearance.

The fibre structure of the chrome tanned sample was similar to that of the sheepskin salted in the wool.

(ii) GOATSKIN.

(*a*) *Fresh Skin.*

Examination of samples cut from different positions in the goatskin showed that in the flank the angle of weave tended to be lower and the fibres rather thinner and more loosely woven than towards the backbone. As in the sheepskin, these differences were not very marked.

(*b*) *In the Hair, Pickled.*

Pickling the goatskin in the hair caused a very marked increase in the fullness of the fibres. The weave pattern of this sample was very regular.

During storage the fibres became progressively thinner and rather more opened up, while the weave pattern became rather less regular and the angle of weave lower. After chrome tanning the fibre structure was comparatively good considering the macroscopical appearance of the pelt, the fibres were full and much opened up, but the fibre weave was rather irregular with open spaces.

(*c*) *In the Hair, Salted.*

Salting the goatskin in the hair caused a very marked shrinkage in the fibres. During storage the skin became so dry that the fibres had a glued appearance at the end of 8 months.

Although the sample had become so dry extensive mould growth was found on the skin after 3 months' storage.

The fibre structure of the chrome tanned sample was poor, the fibres were unevenly opened up and there was a generally stringy appearance suggesting breakdown of the collagen fibres.

(*d*) *Unhaired.*

The fibre structure of the skin after liming was much more opened up and the fibres fuller than in the fresh skin.

(*e*) *Unhaired, Pickled.*

Pickling caused the fibres to become rather fuller and slightly more split up than in the limed skin, the fibres were very full, well opened up and of a high angle of weave for a goatskin (Fig. 6). After storage for

3 months the fibres became more opened up and after storage for 8 months the fibres were very much opened up, with some separation of the fibrils (Fig. 7).

The fibre structure of the chrome tanned sample was similar to that of the goatskin pickled in the hair.

(*f*) *Unhaired, Salted.*

The fibre structure of the fellmongered and salted goatskin (Fig. 8) was very much opened up, loosely woven and of a high angle of weave for a goatskin, and presented a marked contrast to that of the goatskin which had been salted in the hair. On storage, however, the skin lost moisture and the fibres gradually shrunk until after storage for 8 months, as shown in Fig. 9, the fibres were very translucent and layered in appearance, and similar to those of the goatskin salted in the hair.

The fibre structure of the chrome tanned sample was similar to that of the corresponding sample salted in the hair.

The Distribution of the Grease in the Sheepskin and Variations therein During Storage.

(i) Results of Microscopical Examination.

(*a*) *Fresh Skin.*

In the sections stained for fat, fat cells could be seen scattered through the corium and in greater number just below the grain. Deposits of fat were also visible just inside the flesh layer. The fibres of the grain had also taken the fat stain and, as would be expected, the sebaceous glands were deeply stained. The amount of fat in the skin varied; fat occurred in the greatest amount in the kidney area and decreased in amount both in the direction of the shoulder and of the flank. The fat in the fresh skin was largely in globular form. It was probably contained in unbroken fat cells. Fig. 10 shows a typical section of a fresh sheepskin stained for fat.

(*b*) *Skin in Wool, Pickled.*

Immediately after pickling the fat globules were rather less regular in shape than in the fresh skin. The fat was chiefly within the cells and the distribution of the deposits of fat was similar to that in the fresh skin. After storage of the specimen for 6 weeks in the pickled condition, the grease was beginning to migrate from the fat cells.

After 3 months' storage the stained sections were very similar in appearance to those taken from the skin after 6 weeks' storage. After storage for 8 months nearly all the fat had migrated from the cells and the fibres themselves had become stained a light red, indicating that the fat had spread over the fibrils.

(*c*) *Skin in Wool, Salted.*

Fat was obvious throughout the skin, but was particularly large in amount at the junction of the grain and corium and between the layers of fibres near the flesh. The fat in the corium appeared under the microscope as small red spheres and therefore may be considered to be enclosed in the fat cells.

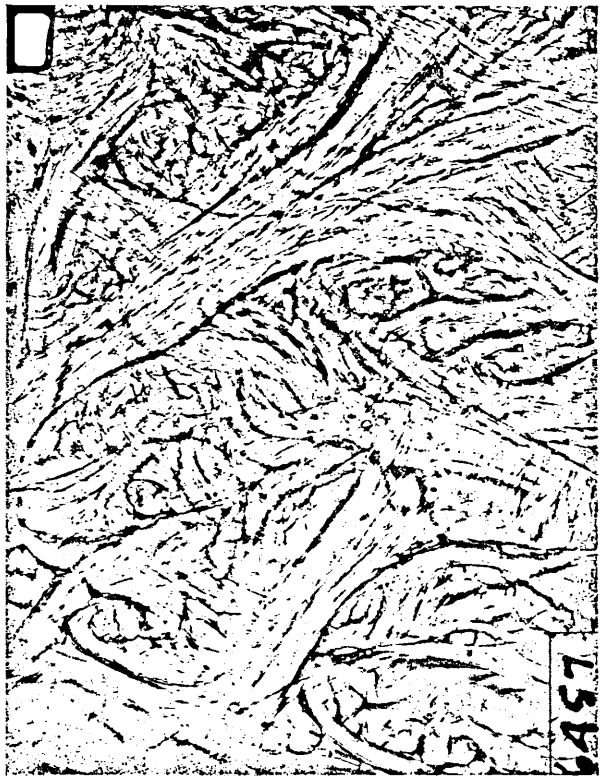

Fig. 6.
Freshly pickled fellmongered goatskin. (× 86).

Fig. 7.
Pickled fellmongered goatskin, after storage for 8 months. (× 86).

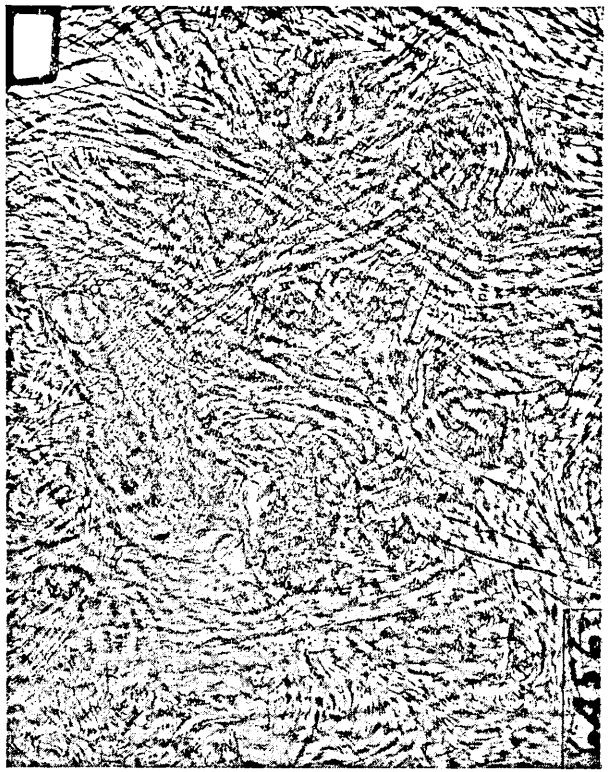

Fig. 8.
Freshly salted fellmongered goatskin. (× 86).

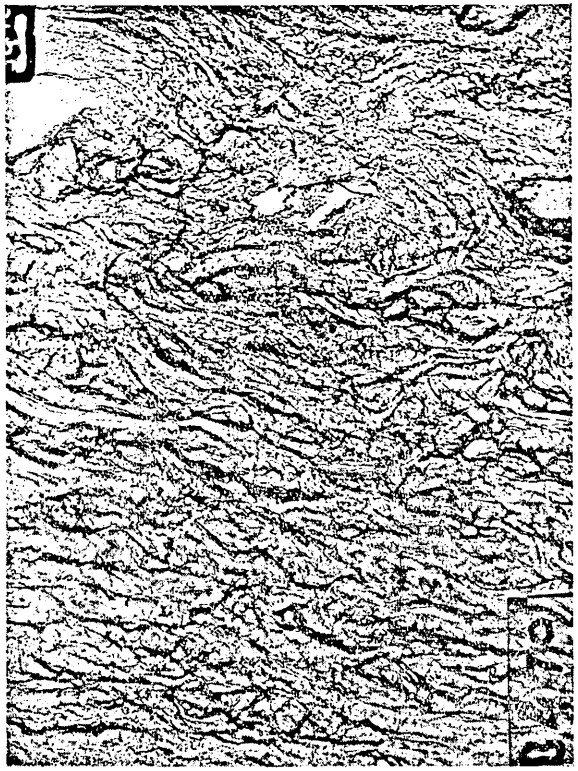

Fig. 9.
Salted fellmongered goatskin, after storage for 8 months. (× 86).

During storage, drying of the samples continued and much of the grease was forced out of the skin. Only a portion of the fat was in globular form after storage for 3 months. After 8 months' storage, examination of the stained section showed that the fat was no longer of globular shape, but had probably been pressed out of the fat cells and was redistributed between and on the fibres throughout the corium (see Fig. 11). Some fat had been pressed out of the skin on to the wool.

(*d*) *Dewooled Skin.*

The distribution of the fat in the limed sheepskin was similar to that in the fresh skin. Most of the fat was still within the cells, but the cells were slightly less spherical than in the fresh sheepskin.

(*e*) *Dewooled Skin, Pickled.*

The distribution of the fat in the freshly pickled dewooled sheepskin was similar to that in the limed samples and also to that in the specimens immediately after pickling in the wool. After storage for six weeks, however, the fat was obviously coming out of the cells. This can be seen in Fig. 12. Not only were the fat globules no longer spherical, but the fibres had a more translucent appearance as if lightly coated with grease. Prolonging the time of storage caused further migration of the fat, until after 8 months all the fibres were lightly coated with grease while irregular deposits of fat were observed scattered through the corium.

In the sheepskin pickled with a deficiency of acid the movement of the grease from the cells was rather slower than from the cells in the well pickled samples. After 8 months' storage, however, most of the grease had migrated from the cells.

(*f*) *Dewooled Skin, Salted.*

After storage of the dewooled sheepskin in the salted condition for 8 months, the fat was chiefly located in the middle of the skin, but had lost its globular form and was largely spread over and between the fibres.

(ii) Results of Chemical Examination.

Tables VIA and VIB give the proportions of grease extracted from the sheepskin by the methods described. The grease contents recorded are calculated on the weight of air-dried salt-free skin. The variations in total grease content of the samples are due to local variations in the skin. From the standpoint of the present investigation, these variations are of less significance than the variations found in the proportions of the total grease contents which were removed in the first extraction with light petroleum. In the fresh skin, between 50 and 60 per cent. of the grease was removed in the first extraction. This proportion was increased to approximately 90 per cent. by pickling, and after storage in the pickled condition for six weeks rose to 97 per cent. and remained constant at 98 per cent. during further storage. These variations must be due to some changes in the fat cells and are probably due to rupture of the reticular walls of the cells, since the

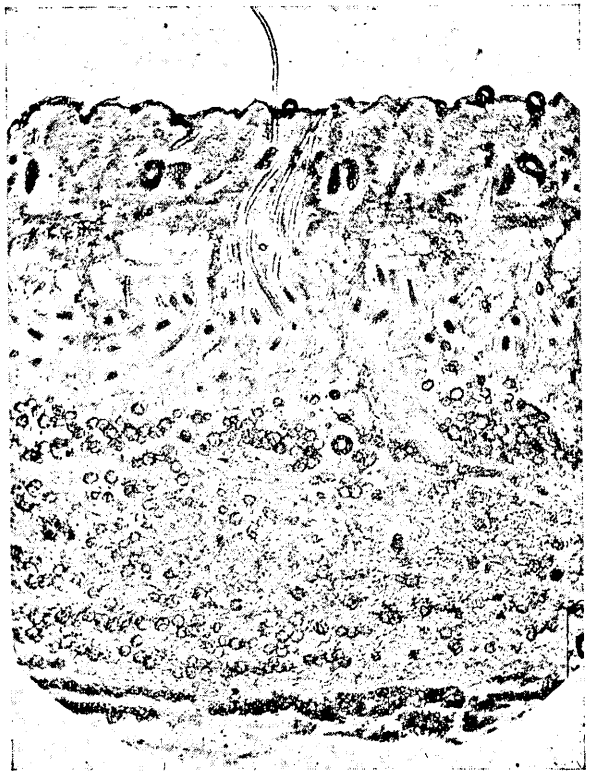

Fig. 10.
Fresh sheepskin. Sampling position 1*x*.
Stained scarlet red. (× 20).

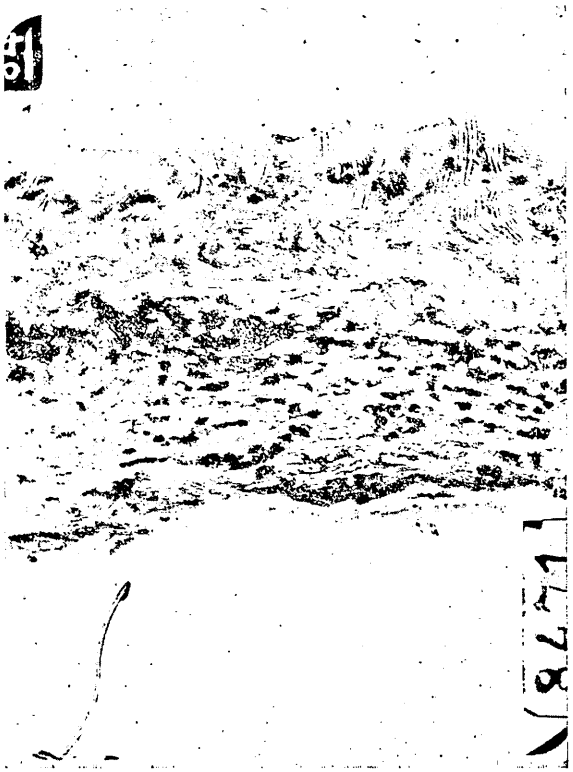

Fig. 11.
Salted sheepskin in the wool, after storage for 8 months.
Stained scarlet red. (× 20).

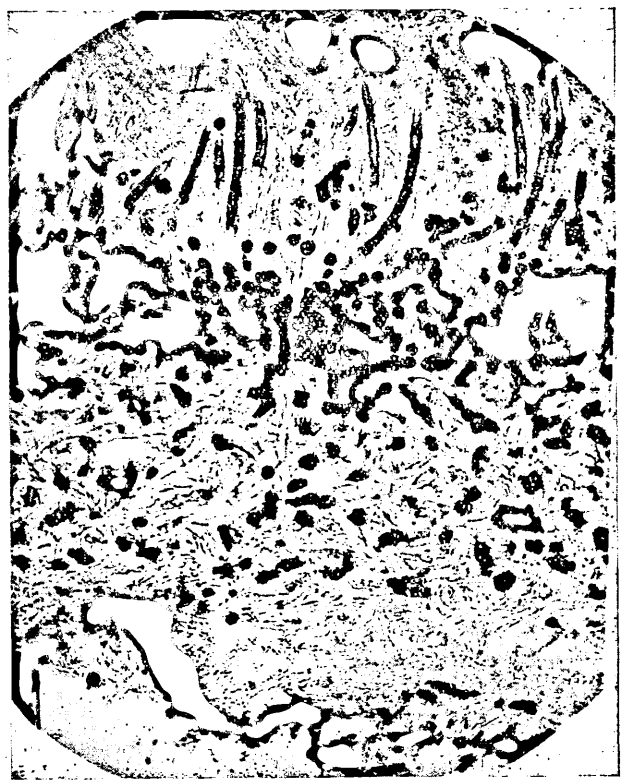

Fig. 12.
Pickled fellmongered sheepskin, after storage for 6 weeks. Sampling position 4*x*. Stained scarlet red. (× 20).

Fig. 13.
Fresh goatskin. Stained scarlet red. (× 20).

TABLE VIa

Grease Contents of Sheepskin—Side in Wool

	PICKLED QUARTER ——g. per 100 g. air-dried, salt free skin—— Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Before treatment (fresh skin)	18·5	10·0	5·6	34·1	54·2
Immediately after treatment...	36·1	4·3	0·9	41·3	87·4
Stored for 6 weeks	42·7	0·1	1·4	44·2	96·6
Stored for 3 months... ...	28·9	0·2	1·2	30·3	95·4
Stored for 8 months... ...	36·3	0·1	0·7	37·1	97·8
	38·6	0·3	0·7	39·6	97·5

	SALTED QUARTER ——g. per 100 g. air-dried, salt free skin—— Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Before treatment (fresh skin)	14·5	5·6	4·9	25·0	58·0
Immediately after treatment...	14·9	7·3	4·4	26·6	56·0
Stored for 3 months... ...	13·6	14·0	8·8	36·4	37·4
Stored for 8 months... ...	10·0	1·6	2·4	14·0	71·4
	29·3	1·2	2·1	32·6	90·0

microscopical examination showed that during storage in the pickled condition the amount of grease which had migrated from the fat cells increased. Jordan Lloyd and Marriott (1935) found that reticular tissue is weakened in solutions of hydrochloric acid at pH values below 2. The pickle liquors used in the present investigation had pH values below 1.0 and would therefore result in weakening of the walls of the fat cells, but since the samples were removed from the liquors after 24 hours and had, after storage,

TABLE VIb

Grease Contents of Sheepskin—Dewooled Side

	PICKLED QUARTER ——g. per 100 g. air-dried, salt free skin—— Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Before treatment (delimed skin)	28·4	9·7	4·1	42·2	67·3
Immediately after treatment...	38·3	0·1	0·8	39·2	97·7
Stored for 6 weeks	50·5	0·1	2·2	52·8	95·7
Stored for 3 months... ...	40·2	0·4	0·7	41·3	97·4
Stored for 8 months... ...	16·0	0·3	0·8	17·1	93·6
	16·5	—	1·1	17·6	93·7
	45·7	0·1	0·5	46·3	98·7

	SALTED QUARTER ——g. per 100 g. air-dried, salt free skin—— Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Immediately after treatment...	43·0	0·9	1·1	45·0	95·6
Stored for 3 months... ...	32·2	0·4	1·9	34·5	93·3
Stored for 8 months... ...	33·6	0·5	1·9	36·0	93·3

pH values of water extracts varying from 2.2 to 4.4, the weakening of the walls would probably be slow, resulting in a gradual increase, during storage, in the number of cells of which the walls had been broken. It appears that extraction of the grease by light petroleum from unbroken fat cells is comparatively slow and that the rupture of the walls of the cells causes an immediate increase in the rate at which the grease can be extracted. The rupture of the cell walls is followed by a migration of the grease from the cells, but this change is not observed immediately after the cell walls have broken, because the movement of the grease out of the cells is not a rapid process.

The analyses of the specimens of sheepskin salted in the wool show that salting caused no immediate change in the rate at which the grease was extracted. After storage for 8 months there was evidence that the grease was more readily extracted. This change, which was less marked than that which occurred in the pickled quarter, was probably due to mechanical breakage of the cell walls by the drying of the specimens which occurred during storage, and the prolonged storage of the specimens in the dried condition (compare Highberger and Moore, 1934, and O'Flaherty and Roddy, 1935). As shown by the microscopical examination this change was accompanied by extrusion of the fat from the cells. Since in the specimens of fresh skin only approximately 50 per cent. of the grease was readily extractable, the drying of the specimens before analysis had a less marked effect on the rate of extraction of the grease than that which occurred during storage, presumably because the fresh specimens were extracted immediately after drying, without intermediate storage.

Fellmongering followed by liming caused a slight increase in the rate of extraction of the grease. The skins were limed without the addition of sodium sulphide and it appears that the duration of contact with the fellmonger's paint was not sufficient to cause extensive breakdown of the walls of the fat cells. It had, however, some action on the walls, since immediately after pickling the rate of extraction of the fat had almost reached its maximum (93 per cent. of the fat removed on the first extraction). Fellmongering and liming followed by pickling, therefore, caused a more rapid breakdown of the reticular walls of the fat cells than did either of these two processes alone. A similar effect was shown by the combination of fellmongering and liming with salting, probably because the preliminary weakening of the cell walls by the alkaline treatment caused them to be more readily broken by the mechanical disturbance of dehydration during and after salting. The microscopical examination of the limed skin confirmed these conclusions, since in both pickled and salted specimens the grease was displaced from the fat cells during storage.

The figures given in Tables VIA and VIB suggest that even after treatment which causes complete liberation of the fat from the fat cells, a proportion of grease, varying between 0.5 and 1.0 g. per 100 g. of air-dried skin, could not be extracted from the skin by light petroleum. This grease probably corresponds to the grease which according to other investigators is combined with the protein. On account of the presence of this fairly constant amount of unextractable grease in the skin, the proportion of the total grease

content which was removed in the first extraction varied, not only according to the treatment of the skin, but also directly with the total grease content of the specimen. This is shown, for example, by the analyses of three specimens of the dewooled pickled quarter after 8 months' storage. From the two specimens which contained approximately 17 per cent. of grease, 93 per cent. of the grease was removed in the first extraction, while 99 per cent. was removed from the specimen which contained 46 per cent. of grease.

In the case of the dewooled sample which was pickled with a deficiency of acid, grease determinations were made on two samples after storage for eight months. The grease contents found were 52.5 and 48.5 per cent. of the air dried skin weight, of which 98 and 99 per cent. respectively were removed in the first extraction. In this case the rupture of the cell walls may have been due not only to the combined action of fellmongering, liming and pickling, but also to the damage to the fibre structure caused by mould growth. Moulds are known to attack the proteins of skins, but it is not known whether both collagen and reticulin, or collagen alone, are attacked. As described above, microscopical examination indicated that the weakening of the walls of the fat cells was slower in the dewooled skin pickled with a deficiency of acid than in that pickled in the best conditions. This may be because it contained a smaller proportion of acid, which would have a slower action on the reticular tissue. After 8 months' storage, however, both microscopical and analytical examination showed that the weakening of the cell walls had become as far advanced as that in the well pickled specimen.

The Distribution of the Grease in the Goatskin, and changes therein during Storage.

(i) Results of Microscopical Examination.

Unlike sheepskin, goatskin has no heavy deposit of fat in the middle layer of the skin. The total amount of fat is generally much lower than in sheepskin and the fat is situated chiefly in the grain and in the flesh layers. The distribution of the grease in a section taken from the middle area of the goatskin can be seen in Fig. 13, where it will be noticed that the grease is chiefly distributed over certain regions in the grain and flesh. A comparatively small number of irregularly shaped deposits of grease were visible in the corium. The distribution of the grease showed no change after liming, pickling or salting and did not appear to alter during storage of the samples.

TABLE VIIa

Grease Contents of Goatskin—Side in Hair

	PICKLED QUARTER —g. per 100 g. air-dried, salt free skin—				
	Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Before treatment (fresh skin)	3·2	0·4	—	4·3	74·4
Immediately after treatment...	3·4	0·1	1·0	4·5	75·6
Stored for 6 weeks	5·4	0·1	0·6	6·1	88·5
Stored for 3 months... ...	8·4	0·1	1·0	9·5	88·4
Stored for 8 months... ...	19·8	0·3	0·5	20·6	96·1

	SALTED QUARTER —g. per 100 g. air-dried, salt free skin—				
	Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Before treatment (fresh skin)	12·5	0·5	1·1	14·1	88·7
Immediately after treatment...	6·2	0·7	1·1	8·0	77·5
Stored for 3 months... ...	2·0	0·2	1·3	3·5	57·1
Stored for 8 months... ...	1·9	0·2	1·3	3·4	55·9

(ii) Results of Chemical Examination.

Tables VIIa and VIIb give the proportions of grease extracted from the goatskin by the methods described.

The grease contents of the samples taken from the goatskin were in general lower than those of the sheepskin. The proportions of the total

TABLE VIIb

Grease Contents of Goatskin—Unhaired Side

	PICKLED QUARTER —g. per 100 g. air-dried, salt free skin—				
	Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Before treatment (delimed skin)	2·8	0·4	1·8	5·0	56·0
Immediately after treatment...	4·1	0·1	0·5	4·7	87·2
Stored for 6 weeks	3·3	0·1	1·3	4·7	70·2
Stored for 3 months... ...	5·8	0·1	0·6	6·5	89·2
Stored for 8 months... ...	2·7	nil	0·6	3·3	81·8

	SALTED QUARTER —g. per 100 g. air-dried, salt free skin—				
	Grease from 1st extraction with light petroleum	Grease from 2nd extraction with light petroleum	Fatty acids from hydrolysis of degreased skin	Total grease	Percentage of total grease removed in 1st extraction
Immediately after treatment...	1·9	0·2	1·3	3·4	55·9
Stored for 3 months... ...	1·6	0·1	1·7	3·4	47·1
Stored for 8 months... ...	1·6	0·3	2·0	3·9	41·0

grease which were removed in the first extractions with light petroleum do not show any regular variation according to the treatment of the sample or the time of storage, but appear to be directly related only to the total grease content of the sample. This agrees with the results of the microscopical examination, which showed that much of the grease in the goatskin was not contained in regularly shaped fat cells.

The goatskin contained between 0.5 and 1.8 per cent. of grease which was not removed by extraction with light petroleum. This is a slightly higher range than that found in the sheepskin, whereas the total grease contents of the goatskin were lower than those of the sheepskin. The proportion of the grease in the goatskin which is readily extractable is therefore lower than that found in the sample of sheepskin after rupture of the cell walls. In the goatskin, this proportion approached 90 per cent. only in the specimens

with the highest grease contents, and exceeded 90 per cent. only in one case, viz. in the specimen with the highest grease content.

The Composition of the Grease in the Samples, and Variations therein During Storage.

(i) Greases Extracted from Sheepskin.

Table VIII records the free fatty acid, unsaponifiable matter and oxidised fatty acid contents of the greases extracted from the sheepskin, expressed as a percentage of the total amount of grease removed in the two extractions with light petroleum. All the greases were soft solids varying from white to pale brown in colour, the darkest in colour being those extracted from the side pickled in the wool.

(*a*) *Free Fatty Acid Contents.*

The increases in free fatty acid contents recorded in Table VIII are not due to the oxidation of the grease (see (*c*) below). They must therefore be due to hydrolysis of the grease (*cf.* Balfe, 1936).

During drying of the samples of fresh skin for analysis, putrefaction took place and the high proportions of free fatty acid found in these greases were probably formed by hydrolysis of the greases by the putrefactive micro-organisms. During storage, the free fatty acid content of the grease increased most rapidly in the specimen salted in the wool, on which mould growth was observed within eight months. The free fatty acid content also reached a high value after fifteen months in the grease in the quarter pickled in the wool, on which extensive mould growth took place. This suggests that the hydrolysis of grease in sheepskins during storage is caused by mould, a suggestion which is supported by the observation that the growth of mould in leather results in hydrolysis of the grease in the leather (Balfe, 1936).

This suggestion is confirmed by the fact that after 8 months' storage the grease in the dewooled specimen which was pickled with a deficiency of acid, and on which mould developed, contained 77.1 per cent. of free fatty acid.

(*b*) *Proportions of Unsaponifiable Matter.*

The occurrence of unsaponifiable matter in the grease is taken as evidence of the presence of grease of the wool grease type, since after saponification the sterols contained in this type of grease can be extracted with light petroleum and are, therefore, returned as unsaponifiable matter by the customary methods of analysis. Whereas the total extractable grease in the skin varied between 10 and 48 per cent., the amount of unsaponifiable matter in the skin only varied between 0.5 and 1.8 per cent., which corresponds to between 1.3 and 4.5 per cent. of wool grease, assuming that wool grease contains 40 per cent. of unsaponifiable matter (Riess, 1936.) This shows that the large variations in the amount of grease in the skin were due to variations in the amount of triglyceride grease, while the amounts of wool grease in the clipped or dewooled skin varied within much closer limits. The amounts of unsaponifiable matter in the extractable grease therefore tended to vary inversely with the amounts of extractable grease in the skin.

TABLE VIII
Analyses of Greases from Sheepskin

	SIDE IN WOOL					
	Pickled quarter			Salted quarter		
	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids
Before treatment (fresh skin)	19·9	3·4	2·4	29·7	5·9	2·6
Immediately after treatment...	2·3	2·8	1·2	5·3	6·0	5·6
Stored for 6 weeks	2·3	—	—	—	—	—
Stored for 3 months... ...	5·3	3·8	—	10·9	—	—
Stored for 8 months... ...	4·2	2·4	1·4	39·9	12·5	5·8
				31·8	3·8	—
				45·1	—	—
Stored for 15 months ...	50·0	—	5·7	—	—	—

	DEWOOLED SIDE					
	Pickled quarter			Salted quarter		
	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids
Before treatment (delimed skin)	0·5	1·7	1·5	—	—	—
Immediately after treatment...	2·6	3·3	2·8	1·5	1·7	1·5
Stored for 6 weeks	4·2	0·9	1·5	—	—	—
Stored for 3 months... ...	4·1	2·2	—	2·8	—	—
Stored for 8 months... ...	—	10·8	5·6	2·2	2·2	1·1
	3·9	1·6	—	3·8	—	—
Stored for 15 months ...	4·9	—	3·3	—	—	—

(*c*) *Proportions of Oxidised Fatty Acids.*

In skin greases, which have iodine values between 35 and 50, it is improbable that any significant oxidation of the grease can take place, on account of the comparatively small proportion of unsaturated fatty acids in the greases. In accordance with this conclusion there is little evidence in Table VIII that the proportions of oxidised fatty acids in the sheepskin greases increase during storage. The oxidised fatty acid contents of the greases, however, vary in the same way as the contents of unsaponifiable matter. This suggests that some of the fatty acids of the sterol ester greases are returned as oxidised fatty acids by the analytical methods employed.

(*d*) *Melting Points and Equivalent Weights of the Fatty Acids.*

The melting points of the fatty acids from the sheepskin greases ranged between 34°C. and 42°C. and their equivalent weights between 270 and 290.

(ii) Greases Extracted from Goatskin.

Table IX records the analyses of the greases extracted from the goatskin. All the greases were soft solids, varying in colour from white to pale brown.

(*a*) *Free Fatty Acid Contents.*

As in the case of the sheepskin greases, the increases in free fatty acid contents recorded in Table IX are probably due to hydrolysis rather than oxidation. The free fatty acid contents of the greases extracted from the goatskin were in general higher than those of the sheepskin greases both in the fresh and in the stored specimens. This suggests that the grease in fresh goatskins normally contains somewhat higher proportions of free fatty acid than the grease in fresh sheepskins. This may be due to the higher propor-

tion, in goatskin grease, of grease derived from the sebaceous glands, since there is some evidence that this type of grease may contain more free fatty acids than that derived from deposits of fat.

In the goatskin pickled in the hair and in both the unhaired specimens of goatskin, the free fatty acid content of the grease increased, during the

TABLE IX
Analyses of Greases from Goatskin

	SIDE IN HAIR					
	Pickled quarter			Salted quarter		
	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids
Before treatment (fresh skin)	14·3	25·6	0·3	5·3	5·4	1·9
Immediately after treatment...	14·1	21·8	2·7	8·1	12·5	1·7
Stored for 6 weeks	12·2	20·0	6·3	—	—	—
Stored for 3 months... ...	20·6	7·8	—	21·1	—	—
Stored for 8 months... ...	26·2	3·4	2·9	29·8 31·0	33·9	5·4
Stored for 15 months ...	10·7	—	7·4	—	—	—

	UNHAIRED SIDE					
	Pickled quarter			Salted quarter		
	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids	% Free fatty acids	% Unsaponifiable matter	% Oxidised fatty acids
Before treatment (limed skin)	11·6	6·7	6·2	—	—	—
Immediately after treatment...	16·7	14·2	3·3	13·7	26·9	—
Stored for 6 weeks	17·4	42·1	—	—	—	—
Stored for 3 months... ...	31·5	5·9	—	29·9	—	—
Stored for 8 months... ...	21·8	16·7	6·0	20·9 16·0	17·1	5·0
Stored for 15 months ...	7·3	—	6·3	—	—	—

first 8 months' storage, more rapidly than that of the greases in the corresponding sheepskins. This is in part due to the fact that the goatskin contained less grease than the sheepskin, so that the formation of the same amount of free fatty acid in both would result in a higher free fatty acid content in the goatskin grease. In the quarter pickled in the hair (which had a pH of water extract of 3.2), and the unhaired salted quarter, the hydrolysis of the grease may have been due to a slow development of mould. In the unhaired pickled quarter, which had a pH of water extract of 2.3, the cause of the slow hydrolysis is obscure; it may be due to a slight development of mould inside the skin, which was not observed on the surfaces, since the salt content and acidity of the sample (see Tables III and IV) were below the limits given for sheepskin by Pleass (1934).

The greatest increase in free fatty acid content took place in the grease in the goatskin salted in the hair, on which, as on the corresponding sheepskin, extensive mould growth took place. The hydrolysis of the grease may therefore in this case be attributed to the action of the mould.

It therefore appears probable that high free fatty acid contents in the grease in goatskins are usually due to the development of moulds in the skin.

TABLE X

Relation Between the Grease Contents of the Goatskin and the Proportions of Unsaponifiable Matter in the Greases

% Grease removed in 1st extraction with light petroleum		% Unsaponifiable matter In grease		In skin
19·8	...	3·4	...	0·7
12·5	...	5·4	...	0·7
8·4	...	7·8	...	0·6
6·2	...	12·5	...	0·8
5·8	...	5·9	...	0·4
5·4	...	20·0	...	1·1
4·1	...	14·2	...	0·6
3·4	...	21·8	...	0·7
3·3	...	42·1	...	1·4
3·2	...	25·6	...	0·8
2·8	...	6·7	...	0·2
2·7	...	16·7	...	0·5
1·9	...	33·9	...	0·7
1·9	...	26·9	...	0·5
1·6	...	17·1	...	0·2

(*b*) *Proportions of Unsaponifiable Matter.*

The variations in amounts of unsaponifiable matter in the greases from the goatskin are probably due to local variations. Table X shows that the amounts of unsaponifiable matter in the skin vary between 0.2 and 1.4 per cent., corresponding to between 0.5 and 3.5 per cent. of wool grease, whereas the total amount of extractable grease in the skin varies between 1.0 and 19.8 per cent. The goatskin, therefore, resembles the sheepskin, in that its content of wool grease varies much less than its content of triglycerides, and the amount of unsaponifiable matter in the grease varies, generally, inversely with the amount of extractable grease in the skin. Since the amount of wool grease in the goatskin is of the same order as in the sheepskin, while the amount of triglyceride grease is in most cases considerably less in the goatskin, the goatskin greases contain, as a rule, higher proportions of unsaponifiable matter than the sheepskin greases.

(*c*) *Proportions of Oxidised Fatty Acids.*

As in the case of sheepskin greases, the iodine values of greases from goatskins indicate that the greases have undergone little oxidation on exposure to air. The figures given in Table IX are in agreement with this but are not sufficiently complete to show if there is a correlation between contents of oxidised fatty acids and unsaponifiable matter such as was found in the sheepskin greases (Table VIII).

(*d*) *Melting Points and Equivalent Weights of Fatty Acids.*

The melting points of the fatty acids from the goatskin greases varied between 35°C. and 42°C. and their equivalent weights between 260 and 290.

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Summary.

1. Samples of sheep- and goatskins, both depilated and in the wool or hair have been stored in the pickled and salted condition for periods up to 26 months. The general condition and the fibre structure were examined at various intervals during storage. The grease content of the samples was also estimated and the triglycerides, free fatty acids, oxidised fatty acids and unsaponifiable matter in the grease estimated.

2. Mould growth was observed on all except the fellmongered and pickled samples after 15 months' storage. All samples deteriorated to some extent after prolonged storage, especially the sheepskin pickled in the wool. The pH value of aqueous extract of this sample had risen from 2.0 to 8.0 during 26 months' storage.

3. During storage in the pickled condition an increase in the opening up of fibres took place due to weakening of the reticular tissue round the fibres and fibrils. During storage in the salted condition, the fibres became shrunken, owing to dehydration of the skins.

4. In sections of sheepskin stained for fat a large number of fat cells could be seen scattered through the corium and in greater numbers just below the grain. In the goatskin there was much less grease; this was chiefly present in the grain.

5. Pickling and liming, processes which tend to weaken the reticular tissue surrounding the fat cells, caused a gradual migration of the grease to take place from the fat cells in the sheepskin during storage and increased the readiness with which the grease could be extracted by light petroleum. In dewooled pickled sheepskins the movement of grease out of the cells could be observed after 6 weeks' storage.

Salting, when preceded by fellmongering and liming, had a similar effect, probably because the preliminary weakening of the cell walls by the alkaline treatment caused them to be more readily broken by the mechanical disturbance of dehydration during and after the salting.

The distribution and ease of extraction of the grease in goatskins was not affected by pickling or liming.

6. The free fatty acid content of the grease increased during storage. This increase was most marked in those samples on which extensive mould growth had been observed. It is suggested that hydrolysis of the grease is due to mould.

7. The amount of unsaponifiable matter in the extractable grease did not vary greatly. Variations in the amount of grease in the skin were, therefore, mainly due to variations in the amount of triglyceride grease.

8. There is little evidence that the proportion of oxidised fatty acids in the grease increases during storage.

THE USE OF SALTS IN THE FINISHING OF SOLE BENDS.

Part I.—The Precipitation of Tannins by Salts.

By J. H. BOWES.

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Part I.—The Precipitation of Tannins by Salts.

By J. H. Bowes.

The modern methods of tanning sole bends in which strong liquors of 150°Bk. or more are used at the end of the tanning process, cause the goods leaving the last liquors to contain a large amount of uncombined material in the interfibrillary spaces. This material tends to creep to the surface during drying causing the leather to dry out a poor colour and to have a cracky grain. Treatment with magnesium sulphate, either alone or with cane sugar or glucose, however, has been found to prevent the occurrence of these defects to a great extent. The wet leather is either drummed with a mixture of magnesium sulphate crystals, and cane sugar or glucose, or immersed for 10 to 30 min. in a saturated solution of magnesium sulphate to which cane sugar or glucose has been added.

One of the most probable functions of the magnesium sulphate is precipitation of the uncombined tanning material present in the interfibrillary spaces of the leather, thus preventing the creeping to the surface during drying, *cf.* Meunier and Roussel[1]. The presence of magnesium sulphate in the leather will also lower the vapour pressure of the water in the leather, and so affect the rate of drying, and the water absorbing properties of the leather.

The function of the cane sugar and glucose generally used in conjunction with magnesium sulphate is less clear. They too will have an effect on the vapour pressure of the water in the leather, and so help to regulate the loss of water during drying, and to increase the water absorptive capacity of the finished leather. It has also been suggested that they prevent the formation of salt spue due to the presence of magnesium sulphate.

This work was originally started in order to find a substitute for magnesium sulphate and sugar in the treatment of sole bends, but it has been extended to cover some of the theoretical considerations involved in the precipitation of tannins by salts, and the effect of sugars and other organic compounds on this precipitation. The effect of these materials on the formation of salt spue (Part II) and the effect of treatment with magnesium sulphate and sugars on the loss of water during drying, and on the uptake of water by leather at different humidities has also been studied (Part III).

I. The Precipitation of Tanning Extracts by Salt Solutions.

EXPERIMENTAL METHOD.

5° and 100°Bk liquors of the following tanning materials were prepared, chestnut, valonia, myrabolam, mimosa, gambier, quebracho, mangrove and eucalyptus*, and a 2.5% solution of tannic acid (80°Bk).

Since the pH value of the solution may influence precipitation by the salt solutions, it was decided that all experiments should be carried out at the same

* Extracts of these materials were kindly supplied by Messrs. Richard Hodgson & Sons, Limited.

pH value, and 3.5 was chosen as a suitable value as it lies in the range met with in practice when leathers are treated with magnesium sulphate.

The pH was adjusted with hydrochloric acid or sodium hydroxide, and the liquors allowed to stand for a week prior to use. Molar and saturated solutions of a number of salts were also adjusted to pH 3.5 using the appropriate acid or alkali.

50 cc. of tan liquor were added to 25 cc. of the molar or saturated salt solutions in tall tubes 3.5 cm. in diameter, and the precipitate allowed to settle for 2 days. With the tannic acid solution it was necessary to add 50 cc. of the salt solution in order to produce a readily measurable amount of precipitate. A control experiment consisting of 50 cc. of tan solution and 25 cc. of water was included for each tanning material.

The nature and amount of the precipitate was observed and the organic matter in the supernatant liquor determined by the Loewenthal permanganate titration method[2], and from a similar titration on the original liquor the amount of organic matter precipitated was calculated. It was not possible to use the Official Method to determine the total solids owing to the retention of a variable amount of moisture by the comparatively large amount of magnesium sulphate and other salts present. In preliminary experiments the relative amount of tans and organic non-tans precipitated was determined by carrying out the Loewenthal titration before and after detanning with hide powder.

EXPERIMENTAL RESULTS.

In preliminary experiments the precipitates formed on the addition of an equal volume of saturated magnesium sulphate solution to 100°Bk liquors of certain of the tanning materials were considered. The amount and nature of the precipitate formed varied with the tanning material. It was obvious that in some cases a considerable amount of magnesium sulphate was carried down with the precipitate ; this was especially noticeable with quebracho, where crystals of the salt could be clearly seen. All the precipitates dried out quickly and showed no tendency to be hygroscopic.

The precipitates formed with chestnut, mangrove, mimosa and valonia were almost completely soluble in water leaving only a very small amount of a dark coloured residue. Evidence obtained later suggests that this residue is probably a magnesium tannate. The precipitates dissolved in alcohol to give a dark brown solution leaving a dirty white residue, which from its appearance and ash content (see Table I) was mainly magnesium sulphate. The ash content of the sesquihydrate of magnesium sulphate is given in Table I for comparison. With mimosa and mangrove, the rather lower ash content indicates the presence of some organic matter.

When the alcoholic filtrates were evaporated to dryness, a dark brown residue was obtained resembling the original tanning material. The ash content of these residues was small (Table I), indicating that this alcohol-soluble portion of the precipitate is not a magnesium salt of the tanning material but the salted out tanning material itself. In confirmation of this the dry residue from the alcoholic filtrate obtained with tannic acid was dissolved in water and Loewenthal and potentiometric titrations made on the

solution. The results obtained were identical with those obtained with a tannic acid solution of the same strengh.

TABLE I.

ASH CONTENTS OF PRECIPITATES
(Weighed as Sulphates)

Liquor	% Ash in alcohol-insoluble material	% Ash in alcohol-soluble material
Chestnut ...	54	2·0
Mimosa	40	0·7
Mangrove ...	43	1·5
Valonia	46	1·1
Tannic Acid ...	50	0·73
$MgSO_4.7H_2O$ ...	49	—

It was concluded from these results that the precipitation of tans by magnesium sulphate is primarily due to salting out and not to the formation of magnesium tannate. The small amount of the precipitate which is insoluble in water and alcohol was considered to be magnesium tannate. This was only a very small fraction of the total precipitate.

The magnesium salts of tannic acid and mimosa tannin were prepared by the addition of sodium hydroxide to solutions of these tannins containing a small amount of magnesium sulphate, and compared with the precipitates formed on the addition of saturated magnesium sulphate to these solutions. Precipitation started at pH 4.0 and increased as more alkali was added up to pH 8.0. The precipitates formed were different in appearance from those obtained by the addition of saturated magnesium sulphate. With tannic acid, the precipitate was more flocculent, did not coagulate, and tended to darken in colour on exposure to air ; the mimosa precipitate also darkened on exposure to air, becoming a dirty violet in colour. Both precipitates were insoluble in alcohol and almost insoluble in water at pH values above 5.0, but on acidification they readily dissolved. The ash contents of the dried samples (weighed as sulphate) were 17.1 and 16.2%, respectively for tannic acid and mimosa, and assuming the molecular weight of these tannins to be 1700[3] this corresponds roughly to the combination of 2 molecules of magnesium with each molecule of tan.

If precipitation of tans with saturated magnesium sulphate involves salting out only, it would be expected that it would cause a rise in the pH of the supernatant liquor owing to the removal of tan from the solution ; with both tannic acid and mimosa however, the pH fell (from 2.96 to 2.82 with tannic acid, and from 3.24 to 2.82 with mimosa). Calculation shows that the formation of even the small amounts of magnesium tannate indicated by the experiments described above, and consequent production of sulphuric acid, could account for these decreases in pH.

The Loewenthal titration was carried out on the original and supernatant liquors from chestnut, mimosa and myrabolams before and after detanning with hide powder. The decrease in " oxidisable " matter due to precipitation is approximately the same as the decrease in " oxidisable " tans, showing that it is the tannins which are mainly precipitated (see Table II). The relation

between the two sets of figures is not precise possibly because the presence of salts affects the combination of the tans with hide powder.

TABLE II.

TANS AND NON-TANS PRECIPITATED FROM TAN LIQUORS BY SATURATED MAGNESIUM SULPHATE.

Expressed as cc. N $KMnO_4$ per 100 cc. 50°Bk. liquor.

	Total oxidisable matter		Oxidisable tans	
	Before precipitation	After precipitation	Before precipitation	After precipitaion
Chestnut	191	148	152	102
Mimosa	190	146	182	127
Mangrove	159	103	146	64

(i) *Precipitation by Molar Solutions.*

These experiments were carried out on chestnut (hydrolysable tannin) and mangrove (condensed tannin) liquors. Only those solutions of salts which could be adjusted to pH 3.5 without appreciably altering the molarity of the solution were used. The amount of material precipitated was small with all salts. The percentage organic matter removed in each case is given in Table III.

TABLE III.

THE PRECIPITATION OF TANNINS BY MOLAR SOLUTIONS OF SALTS.

Salt	Percentage oxidisable matter precipitated from 50°Bk. liquor*	
	Mangrove	Chestnut
WATER (control)	Nil	Nil
SODIUM SALTS—		
Fluoride	–3·2	Nil
Bromide	1·2	–3·7
Sulphate	7·0	5·1
Chlorate	7·6	3·2
Nitrate	8·2	1·8
Phosphate	8·2	5·1
Chloride	14·5	9·2
CHLORIDES—		
Potassium chloride	13·9	8·3
Sodium chloride	14·5	9·2
Calcium chloride	15·2	6·5
Magnesium chloride	17·7	6·5
SULPHATES—		
Sodium sulphate	7·0	5·1
Ammonium sulphate ...	8·9	3·7
Zinc sulphate	11·4	3·7
Magnesium sulphate	12·0	4·1
Aluminium sulphate	35·5	5·5
NITRATES—		
Calcium nitrate	4·5	–3·7
Sodium nitrate	8·2	Nil
Magnesium nitrate	8·9	Nil

*Negative signs indicate that the addition of the salt caused an increase in the oxidisable matter present, *i.e.*, it has a peptising rather than s salting out effect.

In some cases the addition of the salt caused an apparent increase in the amount of oxidisable matter; this was probably due to a solubilising action on the insoluble matter present.

TABLE IV.

PRECIPITATION OF TANNINS BY SATURATED SALT SOLUTIONS.

Salt	Solubility g. anhydrous salt per 100 g. water	Gram molecules per 1000 g. solution	Hydrolysable Tans				Condensed Tans				
			Tannic acid	Chestnut	Valonia	Myrab.	Mangrove	Eucalyp.	Gambier	Mimosa	Quebracho
Calcium nitrate	129·2	2·8	—	–4·5	nil	–2·5	8·5	6·8	nil	1·1	7·9
Sodium acetate	46·5	3·2	—	11·0	nil	–2·5	1·8	–3·7	3·5	2·2	4·0
Sodium fluoride	4·3	1·0	11·2	5·5	nil	nil	9·8	–6·2	5·2	4·4	–2·5
Ammonium sulphate ...	75·4	3·2	10·5	7·5	6·5	–6·9	9·8	3·1	6·4	2·7	2·0
Sodium chlorate	101	4·7	—	7·0	nil	–5·0	19·6	11·1	6·9	4·9	20·8
Sodium sulphate	19·4	0·8	10·5	6·5	nil	–3·1	14·0	11·1	2·9	6·5	4·0
Magnesium nitrate ...	42·3	1·7	22·6	7·0	0·6	–2·5	16·5	9·9	10·4	4·9	26·2
Potassium aluminium sulph.	6·0	0·1	11·5	7·5	1·2	9·4	15·8	10·5	nil	–3·3	nil
Ammonium aluminium „	28·2	0·4	9·8	7·5	1·2	8·2	17·0	9·3	3·5	8·2	2·0
Sodium nitrate	87·9	5·5	nil	10·0	1·2	–4·4	26·2	13·0	8·1	2·7	28·8
Sodium dihydrogen phosph.	85·2	3·7	11·2	7·0	10·1	1·3	29·9	19·1	10·4	nil	42·1
Aluminium sulphate ...	36·4	0·6	24·9	10·5	nil	14·5	29·9	16·1	16·8	0·5	27·8
Zinc sulphate	50·9	1·7	44·8	12·0	nil	2·5	33·5	17·3	8·7	1·6	35·1
Lead nitrate	59·5	1·1	—	15·5	—	1·9	12·2	25·9	6·9	10·9	nil
Magnesium sulphate ...	42·9	1·9	64·5	16·0	11·3	8·8	45·1	23·5	12·7	8·2	49·0
Sodium chloride	36·0	4·6	16·8	11·5	19·0	3·8	36·0	31·5	14·4	9·2	60·3
Calcium chloride	74·5	2·4	76·7	18·0	9·5	18·9	34·8	33·3	15·6	10·9	51·4
Magnesium chloride ...	54·5	2·6	85·1	24·5	22·6	32·7	48·8	42·6	17·9	21·2	60·9
Aluminium chloride ...	69·5	2·3	86·5	39·0	10·1	47·9	65·5	54·3	33·0	41·8	84·1
Lead acetate	44·3	0·9	99·8	88·5	83·3	83·1	51·9	68·5	13·9	69·7	86·0
Water (control)			nil	4	0·6	nil	3·7	2·5	1·7	–3·3	0·5

* Negative signs indicate that the addition of the salt caused an increase in the oxidisable matter present, *i.e.*, it has a peptising rather than a salting out effect.

A higher proportion of oxidisable matter is removed from mangrove than from chestnut by all salts. The effect of the salts was in general the same with both tan liquors, but there were certain exceptions, for instance, sodium sulphate precipitated a greater proportion of organic matter from chestnut than did zinc sulphate, whereas with mangrove the reverse was the case. The effect of different anions on the precipitation was determined by comparing the amounts of organic material removed by a series of sodium salts. Sodium chloride precipitated the greatest amount of organic material while sodium fluoride had a peptising rather than a salting out effect. In general the amount of oxidisable matter precipitated decreased in the order, chlorides, sulphates, nitrates. With mangrove the valency of the cation was found to influence the amount of precipitate, divalent cations having more effect than monovalent cations. This was not so with chestnut, however, where the valency of the cation appeared to have little effect.

(ii) *Precipitation by Saturated Solutions.*

A larger amount of precipitate was formed with the saturated than with the molar salt solutions (Table IV).

The type of precipitate varied with the tanning material, and also to some extent with the salt. Sometimes the precipitate was fine and granular, sometimes flocculent and sometimes sticky in consistency. In a few cases, *e.g.*, eucalyptus, it formed a clot which remained suspended in the solution, and in others an apparently large amount of precipitate was formed at first which subsequently redissolved or settled to a very small bulk; this was especially noticeable with aluminium, magnesium and zinc sulphates.

There appears to be some relation between the viscosity of the tan liquor and the type of precipitate, there being a tendency for a fine precipitate to be formed in those liquors having a high viscosity (see Table V). This is understandable since a high viscosity will tend to slow up the movement of the suspended particles and so hinder further aggregation to form large particles. The rate of settling of the precipitate also varied greatly with the tanning material. The precipitates formed with chestnut, mangrove and eucalyptus liquors settled quickly leaving a clear supernatant liquor, while with the other

TABLE V.

RELATIONSHIP BETWEEN THE TYPE OF PRECIPITATE FORMED WITH SATURATED MAGNESIUM SULPHATE AND THE VISCOSITY OF THE TANNING SOLUTION.

Tanning Material	Type of Precipitate.	Viscosity of 100°Bk. liquor in centipoises at 16°C. (Holmes & Brown[4])
Quebracho	Fine, granular	25·0
Valonia	Fine	25·0
Mangrove	Fine	22·0
Gambier	Coarse, tending to coagulate to	14·5
Eucalyptus	sticky mass	8·3
Mimosa		8·2
Chestnut	Plastic mass, drying to hard solid	3·9
Myrabolam		3·8
Tannic acid		—

tanning materials the precipitate did not settle completely even after several days.

With most salts a large proportion of the oxidisable matter was salted out from tannic acid, quebracho, mangrove and eucalyptus liquors, a fair proportion from chestnut and gambier, and a comparatively small proportion from myrabolam, valonia and mimosa.

The amount of organic matter precipitated and the rate at which it settles is probably related to the average size of the colloidal particles in the solution, the larger particles being more easily salted out. There is some agreement between the ease of salting out of certain tannins and their apparent molecular weight after dialysis as determined by Humphreys and Douglas[3] (Table VI).

TABLE VI.

RELATIONSHIP BETWEEN PERCENTAGE COLLOIDAL MATERIAL PRECIPITATED BY SATURATED MAGNESIUM SULPHATE AND THE MOLECULAR WEIGHT OF THE TANNIN.

Tanning Material	Percentage organic matter precipitated	Molecular weight as determined by Humphreys & Douglas[3]
Tannic acid	64·5	3434
Quebracho	49·0	2421
Mangrove	45·2	—
Eucalyptus	23·5	—
Chestnut	16·0	1545
Gambier	12·7	520
Valonia	11·3	—
Myrabolam	8·8	1917
Mimosa	8·2	1704 & 1570

Quebracho and tannic acid with high apparent molecular weights are the most readily salted out, while chestnut, myrabolam and mimosa with lower molecular weights are precipitated less readily. Gambier, although fairly readily salted out, has a much lower apparent molecular weight than the other tannins. The liquor after dialysis still contained 13% non-tans and it is, therefore, probable that the actual size of the gambier tannin molecule is considerably greater than the average value reported. Working on the assumption that the larger particles are more readily salted out, Stiasny and Solomon[5] and Stather and Schubert[6] have carried out fractional salting out experiments with sodium chloride in order to obtain data on the degree of dispersion of various tannins. The technique used was similar to that used in this investigation and the amount of tan salted out was estimated by the Loewenthal method. In Table VII the figures obtained by these authors using a similar concentration of sodium chloride to that used in this investigation are given. These differ somewhat from those obtained here (the different concentrations of tan used are probably the cause), but in all cases the highest percentage of tan was precipiated from quebracho.

The amount of precipitate and the rate of settling also varied with the salt. The precipitates formed with the following salts generally settled rapidly, and gave a clear, supernatant liquor after two days : lead acetate, aluminium, magnesium, sodium and calcium chlorides, zinc, magnesium and ammonium sulphates, lead nitrate and sodium phosphate. In general the different salts behaved similarly with all tanning materials. Lead acetate, aluminium,

magnesium, calcium, and sodium chlorides, and magnesium sulphate precipitate a large or fairly large proportion of organic matter from all tan liquors. Except in a few cases, zinc sulphate, aluminium sulphate and sodium phosphate also precipitate a fairly high percentage of the colloidal material. The remainder of the salts generally precipitate little material, and in many cases have a peptising effect and prevent deposition of any insoluble matter. Aluminium salts appear to be particularly effective in the salting out of the organic material from myrabolam extracts.

TABLE VII.

PERCENTAGE COLLOIDAL MATERIAL PRECIPITATED BY SODIUM CHLORIDE.

Method	Stiasny and Solomon[5] 8 g. NaCl added to 100 cc. tan liquor	Stather and Schubert[8] 12 g. NaCl added to 100 cc tan liquor	Bowes 50 cc. saturated NaCl solution added to 100 cc. 50°Bk tan liquor (*i.e.*, 8·8 g. salt per 100 cc. mixture)
Tannic acid	—	—	16·8
Quebracho	23·6	24·6‡	60·5
Mangrove	17·8*	—	36·0
Eucalyptus	—	—	31·5
Valonia	—	17·2‡	19·0
Gambier	—	8·9‡	14·4
Chestnut	20·6	20·1‡	11·5
Mimosa	8·3†	22·3‡	9·2
Myrabolam	—	21·8‡	3·8

* liquor contained 6·8% tan. † liquor contained 7·5% tan
‡ liquor contained 3·2% tan

With regard to their ability to salt out the colloidal material in tan liquors, the following salts should be as effective, or nearly as effective, as magnesium sulphate for the treatment of sole bends : magnesium, sodium, calcium and aluminium chlorides, zinc and aluminium sulphates and lead acetate. This, however, is only one factor involved in the choice of a salt for the treatment of sole leather, its effect on the feel and appearance of the leather, its tendency to give a salt spue, and its price and availability must also be considered (see Section IV).

II. The Effect of Sugars and Other Organic Substances on the Precipitation of Tannins by Saturated Salt Solutions.

Cane sugar or glucose are usually added to the magnesium sulphate used in the treatment of sole bends, and the effect of these sugars and other organic compounds on the precipitation of the colloidal material from tan liquors by magnesium sulphate has been studied with the object of throwing some light on their function. Preliminary experiments showed that cane sugar andg lucose, glycerol, ethylene glycol, ethyl alcohol, acetone and urea prevented or diminished the amount of precipitate formed with magnesium sulphate. In those cases where the precipitate was diminished there was considerable delay in its formation. The addition of these substances to tannins which had already been salted out with magnesium sulphate immediately redissolved the precipitate.

The relative effectiveness of these organic substances in decreasing the salting out action of a concentrated magnesium sulphate solution was investi-

gated. Varying amounts of the organic compounds (see Table VIII) were added to 80 cc. of saturated magnesium sulphate solution and the volume made up to 100 cc. In the case of the strongest cane sugar, glucose and urea solutions the volume slightly exceeded 100 cc. The effect of addition of 20 g. of ethyl alcohol or acetone to the saturated salt solution could not be determined since these amounts caused the magnesium sulphate to be precipitated. The pH of the solutions was adjusted to 3.5. 20 cc. of each of these solutions were added to 20 cc. samples of a 12.5% tannic acid solution and to 20 cc. samples of 50°Bk mimosa, chestnut and mangrove liquors. The pH of these liquors was also adjusted to 3.5.

TABLE VIII.

THE EFFECT OF ORGANIC COMPOUNDS ON THE SALTING OUT OF TANNINS BY MAGNESIUM SULPHATE.

Organic compounds added to 80 cc. saturated $MgSO_4$ and the volume made up to 100cc.

Additions to magnesium sulphate solution g.per 100 cc.	Amount of tannin precipitated			
	Tannic acid 12·5%	Chestnut 50°Bk.liquor	Mimosa 50°Bk.liquor	Mangrove 50°Bk.liquor
Nil	+++	++++	+++	++++
50 g. cane sugar ...	—	+	+	+
20 g. cane sugar... ...	—	++	++	+++
10 g. cane sugar... ...	+	+++	++	+++
5 g. cane sugar... ...	+	+++	++	+++
50 g. glucose	—	+	++	+
20 g. glucose	+	+++	++	+++
10 g. glucose	++	+++	++	+++
5 g. glucose	+++	+++	+++	+++
20 g. glycerol	—	+	+	+
10 g. glycerol	—	++	++	++
5 g. glycerol	+	++	++	++
2·5 g. glycerol ...	+	+++	++	++
20 g. ethylene glycol ...	—	+	—	+
10 g. ethylene glycol ...	—	++	+	++
5 g. ethylene glycol ...	—	++	++	++
2·5 g. ethylene glycol...	++	+++	++	+++
20 g. ethyl alcohol ...	——	——	——	——
10 g. ethyl alcohol ...	—	++	+	+
5 g. ethyl alcohol ...	—	++	+++	++
2 g. ethyl alcohol ...	+	+++	+++	+++
20 g. acetone	——	——	——	——
10 g. acetone ...	—	±	+	+
5 g. acetone	+	+	++	+
50 g. urea	—	+	—	+
20 g. urea	—	++	++	++
10 g. urea	+	++	++	+++

It was not possible to obtain quantitative figures by means of the Loewenthal method, for the amounts of precipitate formed, since many of the organic compounds reduced the permanganate under the conditions of the titration and so interfered with the determination. The amount of precipitate was estimated, but owing to the variation in the nature of the precipitate this was difficult, and the results can only be considered approximate.

The effect of the addition of organic compounds was most marked with tannic acid, the addition of even 5 g. of ethylene glycol or ethyl alcohol was sufficient to prevent any precipitation, and the addition of only 2 g. caused

a considerable reduction in the amount formed. Glycerol and acetone had rather less effect, and urea, cane sugar and glucose still less. Similar but less marked results were obtained with the tan liquors.

It has generally been considered that the function of the Epsom salts treatment of sole bends is to precipitate the colloidal material in the tan liquor present in the interfibrillary spaces. The reason for the use of cane sugar or glucose in conjunction with the Epsom salts is not obvious at first, since these substances reduce the effectiveness of the precipitation to a considerable extent. In practice, however, the precipitation of the colloidal material depends not only on the effectiveness of the precipitating solution, but also on the diffusion of this solution into the interfibrillary spaces of the leather. Attempts have, therefore, been made to study the precipitation under conditions more nearly resembling those occurring in leather, namely in capillary tubing.

In the first experiments 3 mm. bore tubes about 4 in. long were used. The salt solution was drawn up into the tube, the end closed with a small piece of plasticine and the tube stood upright. The tan liquor was added to the top of the salt solution by means of a fine capillary tube, care being taken to avoid the formation of a bubble at the junction of the two liquids. The formation of a precipitate, and the rate of diffusion of the tan liquor into the salt was observed. It was found that with saturated magnesium sulphate and chloride solutions a clot of precipitate was formed at the junction of the two liquids which prevented further diffusion. The addition of 20% of cane sugar, glucose, glycerol or ethylene glycol to the salt solution reduced the amount of precipitate, but no clot was formed, diffusion continued and such precipitate as was formed was distributed evenly throughout the tube. With magnesium chloride the presence of cane sugar did not entirely prevent the formation of a clot.

In leather the capillaries are much finer and there is, therefore, more probability of a clot forming. Further experiments were, therefore, carried out in very fine capillary tubing (0.3 mm. bore). A short length of this tubing was attached to a Burroughs Wellcome micrometer-syringe and a small amount of tan liquor drawn into the tube, followed by an equal amount of salt solution. 100°Bk mimosa, chestnut, eucalyptus and mangrove liquors were used for these experiments. With saturated magnesium sulphate a clot was formed at the junction of the two liquors, except in the case of mangrove, where a larger amount of precipitate was formed in bands along the tube. In the presence of cane sugar less precipitate was formed, and this was distributed fairly evenly throughout the tube.

Typical examples of the precipitate formed and their distribution are shown in Fig. 1. The capillary space is shown as a darker line running down the centre of each tube, that occupied by the magnesium sulphate solution is greyish in colour, while that occupied by the tan liquor is considerably darker. The precipitate, when present, shows up as a completely dark area with irregular edges. In the case of magnesium sulphate alone (A) a large clot of precipitate can be seen, and the light colour of the liquor to the right of the clot shows that little further diffusion of the tan liquor has taken place. Magnesium sulphate

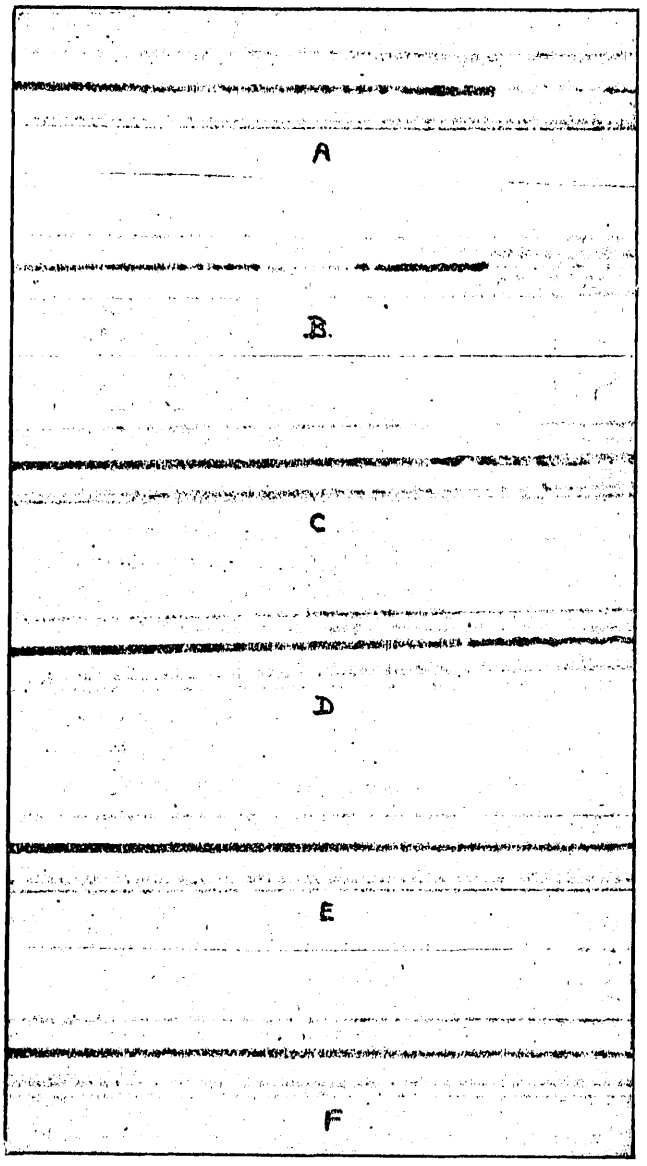

FIG. 1.

Precipitation of tannins in capillary tubes. Magnification × 4.

A	Magnesium sulphate
B	Magnesium sulphate + 0·5% alginate
C	Magnesium sulphate + 20% cane sugar
D	Magnesium sulphate + 20% glucose
E	Magnesium sulphate + 20% glycerol
F	Magnesium sulphate + 20% ethylene glycol.

to which sodium alginate had been added gives a similar clot of precipitate (B). With cane sugar and glucose (C and D) the precipitate is more evenly distributed, while with glycerol (E) and ethylene glycol (F) there is little precipitate formed, the few clots visible being well distributed.

It was thought possible that the sugar did not actually prevent salting out of the colloid particles, but slowed up the precipitation process by increasing the viscosity of the solution, thereby delaying the aggregation of the colloid particles. Experiments were carried out to verify this point. Solutions of magnesium sulphate and the different organic substances of approximately the same viscosity were made up, and the precipitation of 100° Bk mimosa, chestnut and mangrove liquors by these solutions in fine bore capillary tubes (0.3 mm. bore) was examined (see Table IX).

TABLE IX.

THE EFFECT OF VISCOSITY ON THE PRECIPITATION OF TANNING BY MAGNESIUM SULPHATE AND ORGANIC COMPOUNDS.

Substances added to 75 cc. saturated magnesium sulphate solution and volume made up to 100 cc.

Additions to magnesium sulphate solution	Viscosity relative to water	Tan Liquor		
		Mimosa	Chestnut	Mangrove
(1) Nil	2·5	Clot formed, little diffusion	Clot formed, little diffusion	Precipitate in bands, little diffusion
(2) 32 g. cane sugar	7·0	Little precipitate, even diffusion	Some precipitate, fairly even diffusion	Some precipitate, even diffusion
(3) 56 g. glucose ...	6·5	as (2)	as (2)	as (2)
(4) 20 g. glycerin ...	6·7	as (2)	as (2)	Little precipitate, even diffusion
(5) 26 g. ethylene glycol...	6·8	No precipitate, even diffusion	Little precipitate, even diffusion	No precipitate, even diffusion
(6) 15 cc. of 2·5% sodium alginate solution ...	7·0	Fine precipitate, some diffusion	as (1)	as (1)

From these results it may be concluded that viscosity does not play a major part in determining the rate of diffusion of the salt solution into the capillaries or the amount of precipitate formed.

III. The Effect of Treatment in Salt Solutions on the Appearance of the Leather.

A sample of leather prior to the Epsom salts treatment was obtained. Small pieces about 1 in. square were immersed in 20 cc. of a series of different salt solutions (see Table X) for 30 min., rinsed in water, lightly oiled with cod oil and allowed to dry grain uppermost.

The colour of the salt solution after removal of the leather, and the colour of the leather, were noted (Table X).

The colour of the solution after use gives a rough estimate of the tan washed out from the leather during treatment. This depended on the Barkometer strength of the solution used, those greater than 200°Bk, *i.e.*, of higher

Barkometer than the tan liquors from which the leather had been taken, almost completely prevented washing out of the tan.

TABLE X.

IMMERSION OF LEATHER IN SATURATED SALT SOLUTIONS.

(Laboratory experiments)

Salt solution	Colour of solution after immersion of leather	Colour of leather after drying
Magnesium sulphate	Almost colourless	Light
Magnesium chloride		Light
Sodium phosphate		Fairly light
Lead acetate		Very dark
Calcium chloride	Very light brown	Light
Zinc sulphate		Fairly light
Sodium chloride...		Rather dark
Calcium nitrate		Rather dark
Aluminium chloride		Dark
Sodium hexametaphosphate (Calgon) ...	Light brown	Light
Magnesium nitrate		Fairly light
Ammonium sulphate ...		Fairly light
Potassium alum		Rather dark
Sodium sulphate		Rather dark
Sodium nitrate		Rather dark
Lead nitrate		Very dark
Aluminium sulphate	Brown	Fairly light
Ammonium alum		Rather dark
Water		Rather dark
Sodium chlorate		Dark
Sodium fluoride		Dark
Sodium acetate		Very dark

The colour of the dried leathers does not run parallel with the amount of tan washed out. Magnesium sulphate, magnesium chloride, calcium chloride and sodium hexametaphosphate (Calgon), gave a leather of a light colour, although little tan has been removed during treatment. Sodium phosphate, zinc sulphate, magnesium nitrate, ammonium sulphate and aluminium sulphate gave fairly light coloured leathers, while leathers treated with lead salts and sodium fluoride were very dark in colour. The leather samples treated with aluminium salts or alum were inclined to have a yellowish tinge and that treated with zinc sulphate a greyish tinge.

It was considered possible that satisfactory results might be obtained with sodium sulphate to which some other salt had been added in order to increase the Barkometer strength. Mixtures of sodium sulphate with aluminium sulphate, ammonium alum, and sodium chloride were tried (see Table XI). The mixture of sodium sulphate and sodium chloride prevented the tan being washed out, but gave only a fairly light coloured leather. The addition of even a small amount of alum or aluminium sulphate gave the leather a decided yellow tinge. Mixtures of sodium chloride with alum and aluminium sulphate also gave poor results.

The effect of the addition of cane sugar to various salt solutions was investigated (Table XI). A solution of cane sugar alone prevented the tan

bleeding out to a very large extent, and all solutions containing cane sugar were light in colour after use, indicating that little tan had been washed out from the leather.

TABLE XI.

IMMERSION OF LEATHERS IN SALT SOLUTIONS AND IN SALT AND SUGAR SOLUTIONS.

(Laboratory experiments)

Solution	Barkometer °	Colour of solution after use	Colour of leather
Satd. Na_2SO_4 + 10% alum ...	142	Brown	Fairly light, rather yellow
Satd. Na_2SO_4 + 10% $Al_2(SO_4)_3.18H_2O$	150	Brown	Fairly light, rather yellow
Satd. Na_2SO_4 + 20% NaCl ...	216	Very light brown	Fairly light, rather patchy
30% $Na_2SO_4.10H_2O$ + 2% $Al_2(SO_4)_3.18H_2O$	156	Brown	Fairly light, rather yellow
Satd. NaCl + 10% alum ...	205	Light brown	Rather dark, rather patchy
25% NaCl + 50% $Al_2(SO_4)_3.18H_2O$	282	Light brown	Dark, patchy
Water	0	Rather dark brown	Dark, patchy
50% Cane sugar	—	Very light brown	Fairly light
Satd. $MgSO_4$	269	Very light brown	Rather dark
Satd. $MgSO_4$ + 50% cane sugar	331	Very light brown	Fairly light
Satd. Na_2SO_4	109	Brown	Fairly light
Satd. Na_2SO_4 + 50% cane sugar	227	Light brown	Fairly light
Satd. NaCl	198	Light brown	Fairly light, rather patchy
Satd. NaCl + 50% cane sugar	296	Light brown	Light
Satd. $ZnSO_4$	324	Very light brown	Dark, rather grey, spue at edges
Satd. $ZnSO_4$ + 50% cane sugar	390	Very light brown	Fairly light
Satd. $Al_2(SO_4)_3$	210	Brown	Dark, rather patchy
Satd. $Al_2(SO_4)_3$+50% cane sugar	320	Brown	Rather dark
Satd. $MgCl_2$	214	Light brown	Light
Satd. $Mg.Cl_2$+50% cane sugar	308	Light brown	Fairly light, rather patchy
Satd. $MgSO_4$+40% cane sugar	254	Very light brown	Fairly light
Satd. $MgSO_4$+40% glucose ...	234	Brown	Light
Satd. $MgSO_4$+20% glycerin ...	235	Light brown	Fairly light
Satd. $MgSO_3$+20% ethylene glycol	214	Light brown	Fairly light
Satd. $MgSO_4$+0·5% sodium alginate	186	Very light brown	Rather dark, patchy spue at edges
Satd. Na_2SO_4+0·5% sodium alginate	98	Rather dark brown	Rather dark

In all cases, except that of magnesium chloride, the leathers treated in the solutions of salt and cane sugar were of a lighter and more even colour than that of the leathers treated in the corresponding salt solutions alone.

The effect of the addition of glucose, glycerol, ethylene glycol and sodium alginate to saturated magnesium sulphate solutions was also tried. The alginate gave definitely poor results, the leather was dark and salt spue appeared on the edges of the sample after a few days. Of the other samples, that treated in the presence of glycerol was the least good. There was little to choose between the other three samples, but that treated in glucose was perhaps a little lighter in colour.

Discussion.

It is clear from the experiments described here that the precipitation of tannins by saturated salt solutions is primarily due to " salting out " of the colloidal material, and that only very small amounts of the magnesium salt of the tannin are carried down with these precipitates.

It seems safe to conclude that one function of the salts treatment given to sole bends is the precipitation of uncombined colloidal material in the interfibrillary spaces of the leather, thus preventing this from creeping to the surface during drying. Salts which cause the greatest amount of precipitation should, therefore, be the most efficient for this purpose. Magnesium sulphate is the salt most widely used in practice and these experiments show, that with the possible exception of lead acetate, aluminium chloride, magnesium chloride and calcium chloride, it is the most efficient salt with regard to the precipitation of tan. Sodium sulphate, which has been used as a substitute with some success,is less soluble than magnesium sulphate, and even at equivalent concentrations is a less effective precipitant.

The ability to precipitate tans, however, is only one factor involved in the choice of a suitable salt for the treatment of sole leather, its tendency to give a salt spue, its effect on the feel and appearance of the leather, and its price and availability must also be considered. With respect to the colour of the leather, magnesium sulphate and chloride, calcium chloride, sodium phosphate and zinc sulphate were found to be satisfactory. Aluminium salts produced a light, but rather yellow coloured leather.

The presence of sugar and other organic compounds decreases the amount of precipitate formed on the addition of salts, and it was not at first clear why cane sugar or glucose is so widely used in conjunction with magnesium sulphate in practice. As a result of experiments on precipitation in capillary tubes, however, it was concluded that one function of the sugar is to prevent the formation of large clots of precipitate in the capillary spaces near the surface of the leather which will hinder further diffusion of the salt solution into the leather. Although the actual amount of material precipitated is less in the presence of sugar, the precipitate will be more evenly dispersed throughout the leather. Another function of the cane sugar or glucose is to reduce the tendency of the salt remaining in the finished leather to spue on storage. This is further considered in a subsequent paper.

With regard to the theoretical considerations involved in the stabilising action of cane sugar and other organic compounds on the salting out of the colloidal material there are two possibilities :—

(1) A solvent effect—the addition of the organic substance causes the properties of the dispersion medium to approach more nearly to those of an organic solvent and the degree of dispersion of the colloidal particles is thereby increased.

(2) An effect on either the tannin particles or on the ions of the salt which diminishes the salting-out power of the latter.

Considering the first possibility, there is some evidence which suggests that the degree of dispersion of tannins is greater in organic solvents than in water. The apparent molecular weight of gallotannin as determined by various workers is generally found to be lower in organic liquids than in water (Table XII). In a lengthy paper by Navassart[15] dealing with the colloidal

TABLE XII.

MOLECULAR WEIGHT OF TANNIC ACID IN VARIOUS SOLVENTS.

Observer	Solvent		
	Water	Acetone	Glacial acetic acid
Paterno[7]	2643–3700		340
Sabanejeff[8]	1104		1113–1322
Walden[9]		1350–1560	
Kraft[10]	1587–1626	753–763	
Held[11]	714–2383	774–852	441–705
Feist[12]	615–1045		
Iljin[13]		1247–1637	
Brintzinger[14]	1780		
Humphreys and Douglas[3]	3434		

properties of tannins, there is certain evidence derived from measurement of the specific optical rotation of liquors before and after dialysis and observations under the ultramicroscope which indicate that the particle size of the dispersed tannin tends to be smaller in organic liquids than in water.

The characteristic physical property of organic solvents which differeln tiates them from water is their lower dielectric constant. The dielectric co-n stant of water is 79, while that of glycerol is 56, of acetone 27 and of ethy-alcohol 26. The dielectric constant of a 30% aqueous solution of cane sugar is 74 and of a 13% ethyl alcohol solution 72.35. These values are much nearer to that for water than to those representative of organic solvents, but even weaker solutions of cane sugar and of ethyl alcohol have a marked effect in preventing precipitation of the tannins by salts. The action of organic substances cannot, therefore, be explained by the supposition that they give the dispersion medium the properties of an organic solvent. It is possible, however, that the addition of the organic substances does increase the degree of dispersion of the tannins to some extent.

It is possible that in the presence of the organic substances the solvation of the ions of the salt or the tannin particles is altered in such a way that dehydration and consequent precipitation of the tannin micelles does not take place. Jirgensons[16, 17, 18, 19] has published several papers dealing with the

effect of alcohol and other organic compounds on the stability of casein, haemoglobin and albumin sols in the presence of salts. He finds that these substances sensitise the sols at low concentrations, have a stabilising effect at concentrations between 40 and 60% by volume and again sensitise at higher concentrations. The stabilising effect was most marked in the presence of high concentrations of salt. He ascribes this stabilising action to the formation of adsorption complexes between the colloid micelle, the salt and the organic compound, by means of which the hydration, and so the stability of the colloid particle is increased. Viscosity measurements also suggest that the particles in these stabilised protein sols are more strongly solvated than the particles of the sols in water.

It is possible that some similar mechanism is responsible for the stabilising effect of the organic compounds in the experiments under consideration here. There was, however, no point of maximum stability and in all concentrations, even as low as 1%, there was a definite stabilising effect.

It is also possible that the organic substances form complexes with either the tannin particles or the ions of the salt, thereby reducing the salting out power of the latter. Complexes of certain salts with organic compounds containing hydroxyl groups are known (Pfeiffer[20]), for instance :—

Magnesium salts in which the magnesium ion is solvated by one molecule of an alcohol.

A variety of salts in which three molecules of ethylene glycol or glycerol are attached to the metallic ion.

Compounds of sodium chloride and sugar such as $NaCl.C_6H_{12}O._6\frac{1}{2}H_2O$, and $NaCl.C_{12}H_{22}O_{11}.2H_2O$.

The formation of complexes of these types between the salts and the organic compounds may be one of the causes of the stabilising action of these substances.

It is, therefore, probable that the organic substances prevent precipitation by reason of the formation of complexes with the salts or with the tannins or both. The fact that tannins are more highly dispersed in organic liquids may also influence the precipitation to a smaller extent. Whatever the exact explanation may be, it is remarkable that concentrations of alcohol and ethylene glycol as low as 1% have such a marked stabilising effect.

Summary.

1. The precipitation of tannin solutions by saturated magnesium sulphate solution is shown to be mainly due to the salting out of the colloidal tanning material. The precipitates formed consist almost entirely of the tannin in question and only contain a very small amount of the magnesium salt of the tannin.

2. Molar solutions of salts precipitated little organic matter and there was no relation with the Hofmeister series. Saturated solutions precipitated a higher proportion of the organic matter. The type of precipitate varied with the tanning material and to a lesser extent with the salt ; there was a tendency for a fine precipitate to be formed in those liquors having a high viscosity.

The amount of precipitate also varied with the tanning material and the salt. A large proportion of the colloidal material was precipitated from tannic acid, quebracho, mangrove, and eucalyptus liquors, a fair amount from chestnut and gambier, and comparatively little from myrabolam, valonia and mimosa.

The ease of salting out of the different tannins showed some relationship to their apparent molecular weight, quebracho and tannic acid, with high molecular weights and correspondingly large particle size, being the most easily salted out. With regard to their ability to salt out the colloidal material, the following salts should be as effective or nearly as effective as magnesium sulphate for the treatment of sole bends : magnesium, sodium, calcium, and aluminium chlorides, zinc and aluminium sulphates, and lead acetate.

3. Salts producing a light or fairly light coloured leather were magnesium sulphate and chloride, calcium chloride, sodium phosphate, zinc sulphate, aluminium sulphate and ammonium sulphate. Leathers treated with aluminium salts had a slightly yellow colour. Leathers treated in solutions of salts containing cane sugar or glucose were a lighter and more even colour than leathers treated in the corresponding salt solutions alone.

4. The effect of cane sugar and other organic compounds containing hydroxyl groups on the salting out of tans was studied. These substances, especially ethylene glycol and ethyl alcohol, prevented or decreased the precipitation of the colloidal matter. Experiments carried out in fine bore capillary tubes suggest that one function of the cane sugar and glucose used in conjunction with magnesium in the treatment of sole bends is to prevent the formation of large clots of precipitate in the capillary spaces near the surface of the leather which will hinder further diffusion of the salt solution into the leather.

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THE USE OF SALTS IN THE FINISHING OF SOLE BENDS.

Part II.—The Effect of Cane Sugar and Other Organic Compounds on the Prevention of Salt Spue.

By J. H. Bowes.

It has been suggested that one function of the cane sugar generally used in conjunction with magnesium sulphate in the treatment of sole bends is to minimise the danger of salt spue on the finished leather.

The property of a salt which renders it liable to spue is connected with the ability of the saturated solution to show the phenomenon generally known as creep.

Hazlehurst, Martin and Brewer[1] have studied the creep of various saturated salt solutions. They suggest that the main factor governing creep is the ability of the solute to displace the solution from, and to spread over, the supporting surface. The relative ease with which the solution and the solute wet the surface is, therefore, important. It may be concluded from this that the addition of oils, etc., to leather will influence the spue by altering the surface over which the salt must creep in order to reach the surface of the leather. Another factor influencing creep is the crystal form of the solute. Solids which crystallise in needles or rods are found to creep more readily than those crystallising in cubes.

In this investigation, the creep of sodium chloride and sodium sulphate solutions to which varying amounts of cane sugar, glucose and other organic substances had been added, has been studied. The degree to which these salts will spue when present in leather and the effect of cane sugar, etc., on the formation of spue has also been considered. These salts were chosen in preference to magnesium sulphate as preliminary experiments showed that they had a greater tendency to spue formation.

Experimental Method and Results.

Solutions of sodium chloride containing varying amounts of cane sugar, glucose, glycerol ethylene glycol, sodium alginate and urea were made up (see Table I).

The solutions were all adjusted to pH 3.5. 10 cc. of each solution were pipetted into a series of small specimen tubes 3.5 cm. in diameter and 7 cm. high, care being taken to prevent any of the solution touching the sides of the tubes above the level of the liquid. These tubes had previously been well washed with distilled water and rinsed with acetone in order to remove any dirt or grease which might affect the creep. The tubes were left undisturbed and the time at which crystallisation and creep started, and the rate at which the creep proceeded, were noted. The rate of creep is influenced by local variations in conditions of evaporation and on the chance formation of crystals near the sides of the tubes, and hence these experiments can only be considered to give a rough estimate of the extent to which these solutions creep.

In all cases except that of the sodium alginate, the addition of organic compounds either prevented or caused a definite decrease in the extent of creep (see Table I). In any particular series, the extent to which creep was inhibited increased with the concentration of the added organic substance. Glycerol was the most effective in preventing creep, followed by urea, ethylene glycol and cane sugar. Glucose had less effect and sodium alginate tended to encourage creep. The rate of crystallisation was not markedly affected by the addition of the organic compounds, but the size of the crystals tended to be smaller in the solutions containing high concentrations of the organic compounds.

TABLE I.

THE EFFECT OF ORGANIC COMPOUNDS ON THE CREEP OF SODIUM CHLORIDE SOLUTIONS

(Organic Compounds added to 80 cc. saturated NaCl and the Volume made up to 100 cc.)

Additions to sodium chloride solution	Time in days						
	2	4	6	8	10	12	14
Nil	—	c+	++	+++	+++++	+++++	+++++
50 g. cane sugar ...	—	c—	—	—	—	—	—
20 g. cane sugar ...	—	c—	—	—	—	—	—
10 g. cane sugar ...	—	c—	—	—	—	+	++
5 g. cane sugar ...	—	c—	—	—	++	+++	++++
50 g. glucose ...	—	—	c—	—	—	+	++
20 g. glucose ...	—	c—	—	+	++	++	+++
10 g. glucose ...	—	c—	—	+	++	+++	++++
5 g. glucose ...	—	c—	+	+++	++++	+++++	+++++
20 g. glycerol ...	—	c—	—	—	—	—	—
10 g. glycerol ...	—	c—	—	—	—	—	—
5 g. glycerol ...	—	c—	—	—	—	—	—
20 g. ethylene glycol	c—	—	—	—	—	—	—
10 g. ethylene glycol	c—	—	—	—	+	+	++
5 g. ethylene glycol	—	c—	—	—	+	+	++
5 cc. 2.5% alginate	+	++	+++++	+++++	+++++	+++++	+++++
2 cc. 2.5% alignate	—	—	++	++++	+++++	+++++	+++++
50 g. urea ...	—	—	c—	—	—	—	—
20 g. urea	—	—	c—	—	—	—	—
10 g. urea	—	c—	c—	—	—	—	—
5 g. urea	—	—	c+	+++	+++++	+++++	+++++

Significance of symbols (Tables I and II).

— = no creep.

\+, ++, +++, ++++ = intermediate amounts of creep.

+++++ = extensive creep.

c = denotes start of crystallisation.

This experiment was repeated at a later date with similar results except that owing to conditions being less favourable for evaporation a much longer period elapsed before creep started.

The creep of a saturated solution of a salt over a glass surface is not necessarily a measure of the extent to which that salt would spue when present in leather and the creep of sodium sulphate solutions on surfaces more nearly resembling that of leather was, therefore examined. Microscope slides were coated with a film of gelatin, and these films either vegetable or chrome-tanned. These slides, together with untreated slides, were then fixed vertically in specimen tubes containing 10 cc. of the different sodium sulphate solutions to which additions of organic compounds had been made. The extent of creep up the slides was noted as before (Table II).

TABLE II.

THE EFFECT OF ORGANIC COMPOUNDS ON THE CREEP OF SODIUM SULPHATE SOLUTIONS.

(Organic compounds added to 80 cc. saturated sodium sulphate and the volume made up to 100 cc.).

Additions to sodium sulphate solution	Time in days: 4			6			8			10			12		
	Glass	Vegetable tanned	Chrome tanned	Glass	Vegetable tanned	Chrome tanned	Glass	Vegetable tanned	Chrome tanned	Glass	Vegetable tanned	Chrome tanned	Glass	Vegetable tanned	Chrome tanned
Nil	c+	c+	c+	++++	++	+	++++	+++	+	+++++	++++	+	+++++	++++	++
20 g. cane sugar ...	—	—	—	c—	c—	c—	—	—	—	—	—	—	—	—	—
10 g. cane sugar ...	—	—	—	c—	c—	c—	—	—	—	—	—	—	—	—	—
5 g. cane sugar ...	—	—	—	c++	c—	c—	++	—	—	+++	—	—	++++	—	—
20 g. glucose ...	—	—	—	c—	c—	c—	—	—	—	—	—	—	—	—	+
10 g. glucose ...	—	—	—	c—	c—	c—	—	—	+	—	—	+	—	—	+
5 g. glucose ...	—	—	—	c—	c—	c—	+	—	+	+	—	+	+	—	+
20 g. glycerol ...	—	—	—	—	—	—	c—	c—	c—	—	—	—	—	—	—
10 g. glycerol ...	—	—	—	—	—	—	c—	c—	c—	—	—	+	—	—	+
5 g. glycerol ...	—	—	—	—	c+	—	c—	++	c—	—	++	+	—	+++	+
20 g. ethylene glycol	—			c—			—			—			—		
10 g. ethlyene glycol	—			—			—			—			—		
5 g. ethylene glycol	—			—			—			—			—		
20 cc. 2.5% alginate	—	—	—	c—	c—	c—	—	—	—	—	—	—	—	—	—
10 cc. 2.5% alginate	—	—	—	c—	c—	c—	—	—	+	—	—	+	—	—	+
5 cc. 2.5% alginate	c++	c—	c+	++	—	+	++	—	+	++	—	+	++	—	+
20 g. urea	—			—			c+			++			+++		
10 g. urea	—			—			c+			++			+++		
5 g. urea	—			—			c+			++			+++		

The extent of the creep on the glass surfaces was similar to that taking place with sodium chloride solutions in the first experiments, glycerol and ethylene glycol were again the most effective, preventing creep at all concentrations for periods up to 12 days. All except the lowest concentrations of cane sugar, glucose and sodium alginate also prevented creep for the same period.

Alginate was more effective in preventing creep of sodium sulphate than sodium chloride solutions, and urea was less effective. The urea caused the sodium sulphate to crystallise in needles instead of in cubes; as already mentioned, compounds forming needle-shaped crystals creep more readily than those which crystallise in cubes, and it is possible that the altered crystalline form produced by the urea is the cause of the more extensive creep in these solutions. It will be noted that as soon as crystals were observed in these solutions, creep started.

Creep occurred less readily on vegetable and chrome-tanned gelatin films than on glass. The addition of the organic compounds again reduced the extent of creep, their effect being more marked with vegetable than chrome-tanned films. Even the lowest concentrations of cane sugar, glucose and alginate prevented creep on the vegetable tanned films for the period of the experiment and with 5%, glycerol, it was only slight (urea and ethylene glycol were not tested with the vegetable and chrome tanned films). With the chrome tanned gelatin films, cane sugar, prevented creep at all concentrations, and with glucose, glycerol and alginate the creep was very slight.

The formation of spue on leathers which had been immersed in the different salt solutions was observed under laboratory conditions. Preliminary experiments showed that it was difficult to see spue on the rough surface of unfinished leather, finished leather was, therefore, used in these experiments in preference to leather taken prior to the Epsom salts treatment. Samples about 1 in. by 5 in., were immersed in 25 cc. of the salt solutions for 18 hrs., rinsed, left in water for 15 mins. to remove some of the excess salt and then blotted dry. The leathers were left to dry out under laboratory conditions and the extent of the spue noted in each case. Sodium chloride showed a much greater tendency to form a salt spue than sodium sulphate probably owing to its greater solubility, and hence higher concentration in the leather.

Cane sugar was the most effective in reducing spue, glucose, urea, glycerol and ethylene glycol were rather less effective, while sodium alginate appeared to encourage the formation of spue. As in the case of creep, the development of spue was retarded as the concentration of the organic substance was increased.

The relationship between the development of salt spue on leather and creep on a glass surface was only approximate, but when spue formation on vegetable tanned leather was compared with creep on a vegetable tanned gelatin film a good relationship was found. The effectiveness of the organic compounds in preventing creep on the vegetable tanned films then ran parallel to their effectiveness in preventing spue.

TABLE III.

THE EFFECT OF ORGANIC COMPOUNDS ON THE FORMATION OF SALT SPUE ON HEAVY LEATHER AFTER TREATMENT IN SODIUM CHLORIDE SOLUTIONS.

(Organic compounds added to 80 cc. saturated NaCl and the volume made up to 100 cc.).

Additions to sodium chloride solution	Appearance of spue Time in days 4	8	12	25
Nil	++++	+++++	+++++	+++++
50 g. cane sugar	—	—	—	—
20 g. cane sugar	—	+	++	++
10 g. cane sugar	—	+	++	++
5 g. cane sugar	+	++	+++	++
50 g. glucose	—	—	—	—
20 g. glucose	—	++	++	+++
10 g. glucose	++	++	++	++++
5 g. glucose	++	++++	+++	++++
20 g. glycerol	+	+++	+++	+++
10 g. glycerol	+	++	++	+++++
5 g. glycerol	++	++++	++++	+++++
20 g. ethylene glycol	—	+	+	+++
10 g. ethylene glycol	+++	+++	+++	+++++
5 g. ethylene glycol	+++	++++	++++	+++++
5 cc. 2.5% alginate	++++	+++++	+++++	+++++
2 cc. 2.5% alginate	++++	+++++	+++++	+++++
50 g. urea	—	—	—	—
20 g. urea	—	++	++	+++
10 g. urea	+++	++++	+	++
5 g. urea	+++	+++	+++	+++

Significance of Symbols

— = no spue

+ / ++ / +++ / ++++ = intermediate amounts of spue.

+++++ = very much spue

Discussion.

These experiments have shown that it should be possible to counteract the tendency of salts to spue when present in leather by the addition of suitable amounts of certain organic compounds. For this purpose cane sugar or glucose are probably the most satisfactory, since glycerol and ethylene glycol are more expensive and will tend to make the leather rather sticky unless used in small quantities. From a practical standpoint the use of urea is excluded, since it attacks the leather to some extent and tends to darken it.

The leathers examined in these experiments were dried out directly after immersion in the salt solutions, and, therefore, contain a much higher percentage of sodium chloride and sodium sulphate than is likely to occur in practice, and hence have a much greater tendency to spue. The fact, therefore, that in many cases spue was not completely prevented does not indicate that the organic substance in question would not prevent spue under normal conditions. In practice a 5% solution of any of the substances under consideration with the exception of sodium alginate would probably be effective in preventing spue.

Instead of adding cane sugar or glucose to the salt solution as in the vatting process, or in the solid state as in the drumming process, it should be possible to effect an economy in sugar by sponging the leather with, for example, a 5% solution of sugar immediately after the salts treatment, prior to drying out.

TABLE IV.

THE EFFECT OF ORGANIC COMPOUNDS ON THE FORMATION OF SALT SPUE ON HEAVY LEATHER AFTER TREATMENT IN SODIUM SULPHATE SOLUTIONS.

(Organic compounds added to 80 cc. saturated sodium sulphate solution and volume made up to 100 cc.).

Additions to sodium sulphate solution	Appearance of spue Time in days 2	4	6	8	10	12
Nil	+	+	++	+++	+++	++++
50 g. cane sugar ...	—	—	—	—	—	—
20 g. cane sugar ...	—	—	—	—	—	—
10 g. cane sugar ...	—	—	—	—	—	—
5 g. cane sugar	—	—	—	—	+	+
50 g. glucose	—	—	—	—	—	—
20 g. glucose	—	—	—	—	+	+
10 g. glucose	—	—	+	+	++	++
5 g. glucose	—	—	+	+	++	+++
20 g. glycerol	—	—	—	—	—	—
10 g. glycerol	—	—	—	+	+	+++
5 g. glycerol	+	+	+	+	++	+++
20 g. ethylene glycol ...	—	—	—	—	—	—
10 g. ethylene glycol ...	—	—	—	+	+	++
5 g. ethylene glycol ...	—	—	+	+	++	+++
20 cc. 2.5% alginate ...	+	++	+++	++++	+++++	++++++
10 cc. 2.5% alginate ...	+	+	+	+	++	++
5 cc. 2.5% alginate ...	+	+	++	+++	++++	+++++
2 cc. 2.5% alginate ...	+	+	++++	+++++	+++++	+++++
50 g. urea	—	—	—	—	—	—
20 g. urea	—	—	—	—	—	—
10 g. urea	—	—	—	—	—	—
5 g. urea	—	—	—	—	—	—

Significance of Symbols

— = no spue.

+, ++, +++, ++++ = intermediate amounts of spue.

+++++ = very much spue.

With regard to the reasons for the effect of cane sugar and other organic compounds on the formation of spue, it has been suggested that cane sugar and glucose cause the leather to retain moisture so that the salt is held in solution and does not crystallise out. It has been shown, however, that heavy leather immersed in 50% solutions of cane sugar and glucose and dried at 70°F. and 70% R.H. holds no more moisture than control leathers which have not received this treatment.

Hazelhurst, Martin and Brewer[1] suggest that for creep to occur it is necessary for both solid and solution to wet the supporting surface, but that the solid should preferentially wet this surface. The cane sugar and the other organic substance may alter the surface tension between the crystal-solid interface or the solution-solid interface, and so decrease the ability of the crystals and solution to wet the surface over which the creep takes place. No data on this point is available, however.

It was considered possible that some information might be gained by measurement of the contact angle between glass and the salt solution, and glass and the salt plus sugar solution, this being a measure of the degree to which the glass is wetted by these two solutions. The contact angle was so small in both cases that measurements were impossible, but it was obvious that the addition of sugar to the salt solution had little or no effect on the contact angle.

The effect of the organic substances may also be due to the fact that these tend to form complexes with salts[2] which may lead to alteration in the wetting properties of the solute in relation to the supporting surface.

Summary.

1. The formation of salt spue on leather is related to the ability of a saturated solution of the salt in question to exhibit the phenomenon generally known as creep.

2. The addition of organic compounds such as cane sugar, glucose, glycerol, ethylene glycol and urea, retard or prevent the creep of saturated solutions of sodium chloride and sulphate on glass, and of sodium sulphate on vegetable and chrome-tanned gelatin films.

3. Sodium sulphate "creeps" less on vegetable and chrome tanned gelatin films than on glass.

4. There is a relationship between spue formation on vegetable-tanned leather and creep on a vegetable-tanned gelatin film.

5. Sodium chloride has a greater tendency to form a salt spue on vegetable-tanned leather than sodium sulphate.

6. Of the organic compounds used cane sugar was the most effective in preventing spue formation, while sodium alignate appeared to encourage spue formation.

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British Leather Manufacturers' Research Association,
1/6, *Nelson Square, London, S.E.*1.

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THE USE OF SALTS IN THE FINISHING OF SOLE BENDS.

Part III.—The Effect of Magnesium Sulphate, Cane Sugar and Glucose on the Drying of Leather and on the Water Content of Leathers at Different Relative Humidities.

By J. H. Bowes and G. O. Morse.

Various reasons for the use of magnesium sulphate and cane sugar or glucose in the treatment of sole bends have already been considered.[1, 2, 3]

It is frequently stated that the presence of magnesium sulphate and sugar, especially glucose, in leathers affects the rate of drying, and tends to cause the leathers to take up water and to become soft when exposed to moist conditions, or alternatively to retain moisture and remain flexible under dry conditions. This statement probably originates from the observation that American leathers, which generally contain a high percentage of magnesium sulphate and glucose compared with English leathers, tend to become soft when imported to the more humid atmosphere of this country, while on the other hand, English leathers exported to America tend to crack under the dry conditions experienced there.

The theoretical explanation of these observations probably lies in the fact that the presence of magnesium sulphate, cane sugar or glucose tends to reduce the vapour pressure of the moisture in the leather. Under any given conditions, the rate of evaporation from a free surface is directly proportional to the difference between the aqueous vapour pressure at the surface of the

leather and the partial vapour pressure of water in the surrounding atmosphere, *i.e.* :—

$$\frac{dw}{dt} = k(p_w - p_a)$$

where p_w =effective aqueous vapour pressure at the surface
p_a =partial pressure of the aqueous vapour in the surrounding atmosphere.
w =weight lost in time t.

The constant k is dependent on the velocity of the air flow over the surface, *i.e.*, on the rate of removal of the layer of air, adjacent to the surface, which becomes saturated with the evaporating moisture.

With a material like leather, other factors affecting the vapour pressure at the surface are involved, such as :—

(i) The physical structure of the leather.
(ii) The presence of water physically or chemically combined with the protein or tannin, the effective vapour pressure of which will be definitely lower than that of free water.
(iii) The presence of dissolved solids.

The presence of uncombined tans and non-tans in leather will tend to reduce the effective vapour pressure at the surface of the leather and the presence of magnesium sulphate, cane sugar and glucose, substances having small molecular weights compared with the tannins, would be expected to reduce the effective vapour pressure still further. Magnesium sulphate and sugars, by reason of their hygroscopic properties, will also tend to cause the leather to take up moisture from damp atmospheres.

Magnesium sulphate may also have indirect effects on the vapour pressure, since it causes precipitation of the tan and tends to remove the water solubles from the surface.

In this investigation experiments have been carried out to determine the effect of treatment of the leather in solutions of magnesium sulphate, cane sugar and glucose, on the rate of drying, and on the water absorption of the leathers at different relative humidities.

I. The Effect of Magnesium Sulphate, Cane Sugar and Glucose on the Drying of Leather.

(i) Experimental Method.

Two samples of leather, about 2 in. by 3 in., taken from a shoulder range, prior to the usual Epsom Salts treatment, were immersed in each of various solutions of magnesium sulphate, cane sugar and glucose for 18 hrs.

The samples were then removed, rinsed in water, blotted free from surface moisture, weighed and hung up to dry under constant conditions of temperature and humidity. The leathers were weighed at intervals during drying until they were constant in weight. The moisture contents of the leathers were then determined by cutting up about 2 g, of the leather into small pieces and drying for 8 hrs. at 100°C in a vacuum oven. It was realised that under these conditions the leathers do not necessarily lose every trace of

moisture, especially those containing magnesium sulphate. (Experiment has shown that this salt retains the equivalent of two molecules of water when dried under these conditions). After this period of drying, however, the leathers were approximately constant in weight. From the weights obtained and the weights of the samples recorded during drying, the moisture content of the leathers at each stage was calculated the values being expressed as g. of water per 100 g. oven-dried leather.

(ii) Experimental Results.

In the first experiment samples of leathers were immersed in the solutions listed in Table I and dried at 70°F and 70% R.H. The water contents of the leathers during drying are given in Table I, and in Fig. 1 the moisture lost is plotted against time. Each value is the mean for two samples ; the values of duplicate samples did not differ by more than 10%.

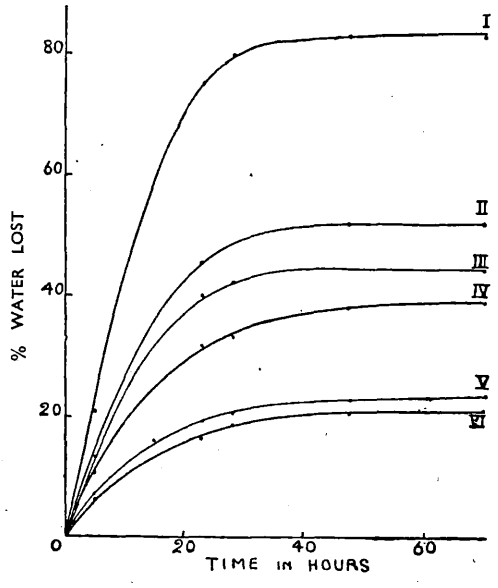

Fig. 1.

Loss of water from leathers during drying after immersion in solutions of magnesium sulphate, cane sugar and glucose (expressed as g. water lost per 100 g. dried leather).

I. Untreated.
II. 50% glucose.
III. 50% cane sugar.
IV. Saturated magnesium sulphate.
V. Saturated magnesium sulphate plus 50% sugar.
VI. Saturated magnesium sulphate plus 50% glucose.

The water content of the samples at the commencement of drying was considerably reduced by treatment in all the solutions, especially by those containing both magnesium sulphate, and cane sugar or glucose. This dehydrating action is probably due to the higher concentration of these solu-

tions compared with that of the tan solution contained in the spaces of the leather, with the result that water tends to pass from the leather to the solution in order to equalise the concentrations. It was found in other experiments that the specific gravity of saturated salt solutions was appreciably lowered by the immersion of leather, indicating that water had passed from the leather into the solutions. The samples all dried to an approximately constant weight in 70 hrs. and the final water contents of the samples were not very different; the sample treated with saturated magnesium sulphate solution had a rather higher and the samples treated with cane sugar and glucose a rather lower final water content than that of the control. Owing to the much lower initial water content of the treated samples at the commencement of drying, the amount of water lost by these during drying was much less than that lost by the control.

TABLE I.

WATER CONTENT OF LEATHERS DURING DRYING.
(g. moisture per 100 g. dried leather).

Treatment*	Time of drying (hours)						g. water lost during drying
	0	5	23	28	48	70	
Water	102·9	88·8	24·7	22·0	19·9	19·7	83·1
Saturated $MgSO_4$	64·9	51·2	33·3	30·9	28·7	26·6	38·3
50% Cane sugar	59·9	49·2	19·7	17·9	15·5	15·6	43·6
50% Glucose	67·5	50·5	22·2	18·9	15·4	15·5	51·2
Saturated $Mg.SO_4$ plus 50% cane sugar	43·3	35·3	24·4	22·9	19·7	19·0	23·7
Saturated $MgSO_4$ plus 50% glucose	40·1	33·0	24·1	22·5	18·9	19·2	20·3

* Percentages refer to concentration in solution.

In the first stages of drying, water was lost at a more or less steady rate, but this was soon followed by a period during which the rate of drying gradually diminished. It is during the first stage of drying when the loosely held water is being removed, and the rate of evaporation is mainly determined by the effective vapour pressure of the moisture in the leather that any effect of magnesium sulphate or sugars on the rate of drying should be apparent. In the latter stages of drying such factors as the rate of diffusion of the water to the surface of the leather, and the extent of the combination of the water with the protein and the tan are the governing factors.

A further experiment (Experiment II) was, carried out in which the leathers were weighed more frequently during the early stages of drying. In this case drying was carried out at 70°F and 60% R.H. In Fig. 2 the water lost during the first 8 hrs. expressed as a % of the oven dry leather is plotted against time, and the water lost per hr. is given in Table II.

From these results it is obvious that the treatments had a marked effect on the rate at which water was lost, as well as on the total amount lost. Saturated magnesium sulphate, either alone or in conjunction with sugars reduced the rate by approximately half and treatment in cane sugar and glucose had rather less effect.

It may be concluded that the treatment of leathers in magnesium sulphate and sugars reduces the rate of drying by reason of the effect of these substances on the vapour pressure of the moisture in the leather. The extent of the lowering of the vapour pressure of the water in the leather by magnesium sulphate, etc., obviously depends on the amount of these compounds present. An experiment was, therefore carried out using a wider range of solutions so that the final concentrations in the leather would be varied. (Experiment III).

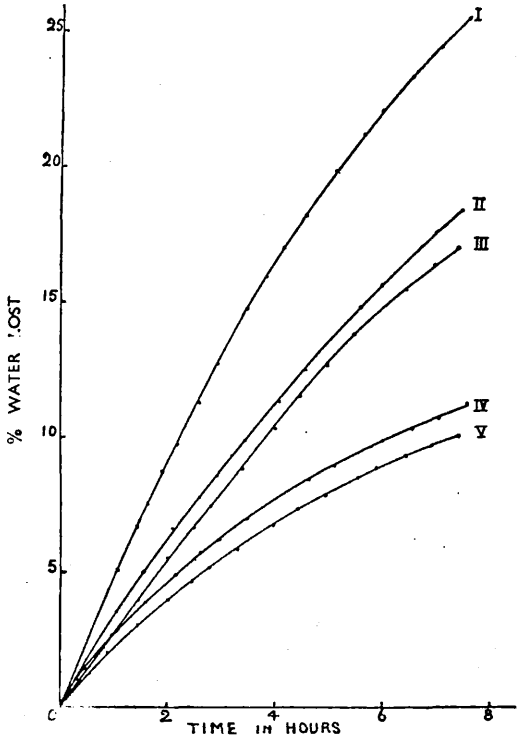

FIG. 2.

Loss of water from leather during first stages of drying after immersion in solutions of magnesium sulphate, cane sugar and glucose (expresssed as g. water lost per 100 g. dried leather).

I. Untreated.
II. 50% glucose.
III. 50% cane sugar.
IV. Saturated magnesium sulphate.
V. Saturated magnesium sulphate plus 50% cane sugar to glucose.

The leathers were dried at 20°C. and 38 to 45% R.H. The results obtained are given in Table III. The equilibrium water content of the samples treated in magnesium sulphate was this time only slightly higher than that of the other samples. The lower humidity was probably the reason for the smaller difference.

TABLE II.

WATER LOST PER HOUR DURING THE INITIAL STAGES OF DRYING.

Treatment.	g. water lost per hour.
None	3·89
50% cane sugar	2·37
50% glucose	2·54
Saturated magnesium sulphate	1·83
Saturated magnesium sulphate plus 50% cane sugar ...	1·78
Saturated magnesium sulphate plus 50% glucose ...	1·49

All the treatments again caused a decrease in the total amount of water lost during drying, but the effects were less marked than before. Treatment in lower concentrations of magnesium sulphate and sugars gave substantially the same results as treatment in the more concentrated solutions.

TABLE III.

WATER CONTENT OF LEATHERS DURING DRYING.

(g. water per 100 g. dried leather).

Treatment*	Time of drying (hours) 0	6	22	30	46	54	g. water lost during drying.
Water	84·5	54·2	15·6	13·2	12·7	12·8	71·7
Tan liquor (100°Bk) ...	87·9	63·7	17·4	13·5	13·5	13·4	74·5
50% Cane sugar	80·5	54·1	18·2	15·4	12·2	12·6	67·9
20% Cane sugar	81·2	58·3	18·8	14·9	13·6	13·6	67·6
50% Glucose	73·9	53·1	20·2	14·1	11·7	11·9	62·0
20% Glucose	77·4	49·7	15·4	12·7	12·8	12·4	65·0
Saturated $MgSO_4$ ...	77·3	52·0	19·1	16·1	15·2	15·3	62·0
Saturated $MgSO_4$ plus 50% cane sugar	69·8	50·5	19·1	16·4	14·2	14·4	55·4
Saturated $MgSO_4$ plus 20% cane sugar	70·8	49·0	19·2	16·1	14·7	14·6	56·2
Saturated $MgSO_4$ plus 50% glucose	75·4	54·6	20·2	16·1	13·9	14·0	61·4
Saturated $MgSO_4$ plus 20% glucose	72·2	50·1	20·1	17·6	14·1	14·4	57·8
5% $MgSO_4$†	79·2	54·5	19·5	15·2	14·2	14·6	64·6
5% $MgSO_4$† plus 20% cane sugar	75·9	51·1	17·4	14·2	13·0	13·2	62·7
5% $MgSO_4$† plus 20% glucose	74·7	51·2	20·0	15·2	13·1	13·1	61·6

* Percentages refer to concentration in solution.
† % anhydrous magnesium sulphate in solution.

II. The Effect of Magnesium Sulphate, Cane Sugar and Glucose on the Moisture Content of Leathers at Different Relative Humidities.

(i) EXPERIMENTAL METHOD.

The absorption of water by the samples of leather treated in the experiments described in the last section was determined at different relative humidities.

Samples of leather from the previous experiments were placed in desiccators over water (approx. 100% R.H.), saturated sodium chloride solution (85% R.H.) and saturated potassium chloride solution (75% R.H.), and the pieces removed and weighed at intervals until they were constant in weight. Air was drawn very slowly, first through a wash bottle of the solution in question and then through the solution in the bottom of the dessicator. In this

way it was possible to regain the required humidity rapidly after opening the dessicator in order to remove the samples for weighing.

The samples exposed to the atmosphere of 85% R.H. were removed as soon as they had reached equilibrium, and placed in a desiccator over water.

Samples of magnesium sulphate, cane sugar and glucose and mixtures of these substances in equal parts were also exposed to atmospheres of 85, 90 and 100% R.H. in order to determine the relative amount of moisture taken up by these materials. The mixtures of magnesium sulphate and sugars were damped and dried in air before exposure in order to reproduce the conditions occurring in dried out leather.

Experimental Results.

(i) The Absorption of Water by Leathers.

The water contents at different relative humidities of the samples from both experiments are given in Table IV.

TABLE IV.

Water Content of Leathers at Different Relative Humidities.
(Expressed as g. water per 100 g. dried leather).

Treatment*	40% R.H.	70% R.H.	75% R.H.	85% R.H.	100% R.H.
Leathers from Experiment I—					
Water	—	19·8	—	—	29·2
Saturated $MgSO_4$	—	26·1	—	—	60·2
50% Cane sugar	—	15·5	—	—	41·9
50% Glucose	—	15·4	—	—	45·6
Saturated $MgSO_4$ plus 50% cane sugar	—	19·6	—	—	49·4
Saturated $MgSO_4$ plus 50% glucose	—	19·8	—	—	52·9
Leathers from Experiment III—					
Water	12·8	—	19·6	21·8	34·0‡
Tan liquor (100° Bk)	13·4	—	21·1	21·3	38·5
Saturated $MgSO_4$	15·3	—	24·8	24·2	56·4
50% Cane sugar	12·6	—	19·8	26·7	44·7
20% Cane sugar	13·6	—	21·8	21·4	—
50% Glucose	11·9	—	20·4	26·0	46·8
20% Glucose	12·4	—	19·6	18·1	41·1
Saturated $MgSO_4$ plus 50% cane sugar	14·4	—	22·5	34·7	56·2
Saturated $MgSO_4$ plus 20% cane sugar	14·6	—	23·6	26·9	52·9
Saturated $MgSO_4$ plus 50% glucose	14·0	—	22·5	34·6	62·1
Saturated $MgSO_4$ plus 20% glucose	14·4	—	26·8	27·8	58·8
5% $MgSO_4$†	14·6	—	22·0	23·2	52·8
5% $MgSO_4$† plus 20% cane sugar	13·2	—	21·9	24·3	47·5
5% $MgSO_4$† plus 20% glucose	13·1	—	22·2	24·9	45·3

* Percentages refer to concentration in solution.
† % anhydrous magnesium sulphate.
‡ Equilibrium not reached. Water content after 20 days exposure.

At 75% R.H. the samples reached equilibrium in 7 days. The water contents of all the samples were of the same order, that of the controls being only very slightly less than that of the treated samples.

At 85% R.H. equilibrium was again reached in about 7 days. Immersion in the various solutions had a definite effect on the amount of water taken up. The effect of immersion in saturated magnesium sulphate and 50% cane sugar or glucose was particularly marked; these samples contained 34% water

compared with only 21% in the controls. Samples immersed in saturated magnesium sulphate and 20% cane sugar or glucose or 50% solutions of the sugars alone also had high water contents (26 to 28%). The water contents of the remainder of the samples were of the same order as that of the controls.

At 100% R.H. the samples treated in Experiment I reached equilibrium in 20 days. By this time the samples which had been immersed in saturated magnesium sulphate and 50% cane sugar or glucose were completely saturated with moisture and drops of water were visible on the surface. The sample which had been immersed in saturated magnesium sulphate contained the most moisture (60%), the rest of the treated samples contained between 40 and 50%, while the controls contained only 30%.

The leathers from Experiment III which had first been exposed at 85% R.H. and then transferred to an atmosphere of a 100% R.H. did not reach equilibrium before the experiment had to be discontinued owing to the extensive growth of mould on the samples. The control samples did, however, reach equilibrium in this time (20 days). The samples treated in saturated magnesium sulphate, either alone or in conjunction with 50% cane sugar or glucose or 20% glucose, contained between 56 and 62% water. The remainder of the treated samples contained between 45 and 52% as against 32 to 38% in the controls. Reduction of the concentration of magnesium sulphate to 10% and of the sugars from 50 to 20% was accompanied by a decrease in the amount of water taken up, but the decreases were not as large as might have been expected. The samples which had been immersed in solutions containing glucose took up rather more water than samples which had been immersed in the corresponding cane sugar solutions.

(ii) The Absorption of Water by Magnesium Sulphate, Cane Sugar and Glucose at Different Relative Humidities.

At 85% R.H. none of the substances absorbed much water; magnesium sulphate and cane sugar, either together or separately, absorbed 0.3%, glucose alone absorbed 5%, and magnesium sulphate and glucose absorbed about 10%. At 90% R.H. the cane sugar and glucose alone took up about 75% of water (see Fig. 3), but the magnesium sulphate alone took up practically none.

It is interesting to note, however, that the mixtures composed of 50 g. magnesium sulphate and 50 g. cane sugar or glucose not only took up more water than 100 g. of the sugars alone, but took it up more rapidly. This can be explained on the assumption that at 90% relative humidity magnesium sulphate does not take up appreciable quantities of moisture, but that its saturated solution does. The vapour pressure of magnesium sulphate ($MgSO_4.7H_2O$) at 25.7°C is 12.29 mm. and that of a saturated magnesium sulphate solution at 20°C is 5.95 mm. (Mellor[3]). It was also confirmed experimentally that a saturated solution of magnesium sulphate took up considerably more moisture than magnesium sulphate crystals at the same relative humidity. In the presence of cane sugar or glucose, the magnesium sulphate dissolves in the moisture taken up by the more hygroscopic sugar and forms a saturated solution, the vapour pressure of which is lower than that of the solid sulphate.

The fact that although magnesium sulphate did not take up a significant amount of water at 85% R.H., leathers containing magnesium sulphate took up considerably more than the control, can be explained on the same basis. Presumably the magnesium sulphate dissolved in the moisture absorbed by the leather itself and a saturated solution of the salt was formed. For the same reason leathers containing sugar as well as magnesium sulphate took up more water than leathers containing similar amounts of either of these substances separately because the sugar, by reason of its own hygroscopic properties, caused more water to be taken up and hence more magnesium sulphate dissolved to form a hygroscopic solution.

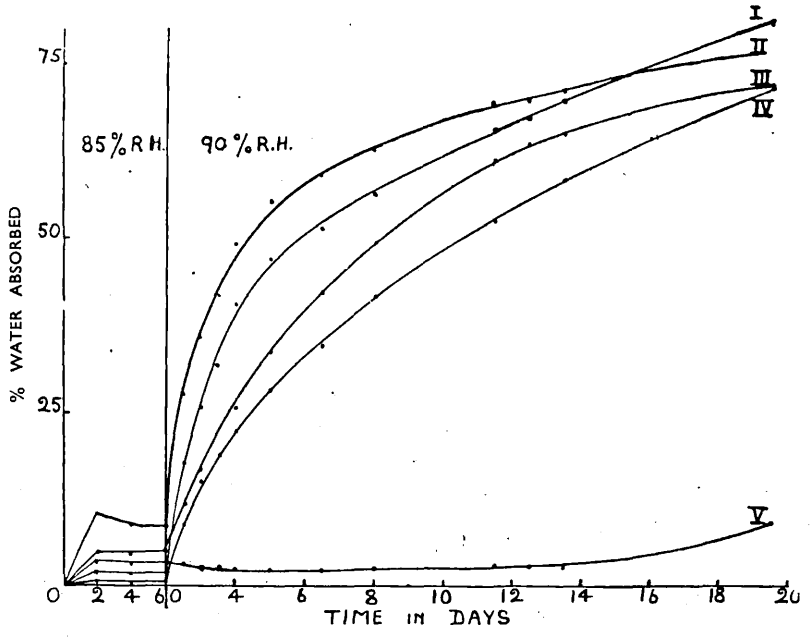

FIG. 3.

Absorption of water by magnesium sulphate, cane sugar and glucose at 85 and 90% R.H. (expressed as g. moisture taken up per 100 g. air dry material).

I. Magnesium sulphate plus cane sugar.
II. Magnesium sulphate plus glucose.
III. Glucose.
IV. Cane sugar.
V. Magnesium sulphate.

(iii) THE ASH CONTENT OF LEATHERS TREATED WITH MAGNESIUM SULPHATE.

The ash content of several of the treated samples was determined to gain some idea of the amount of magnesium sulphate taken up, it being assumed that any ash in excess of that present in the control was magnesium sulphate. The values for the sulphated ashes are given in Table V. In Experiment I the ash content of the sample treated in magnesium sulphate alone is considerably higher than that of the other samples. This offers an explanation

of why this sample took up most water in this experiment and more than the corresponding sample in Experiment III.

In the samples treated in magnesium sulphate alone, the salt is mainly located in the grain layer while in those treated in magnesium sulphate and cane sugar or glucose, the magnesium sulphate is more evenly distributed throughout the leather.

TABLE V.

ASH CONTENT OF LEATHERS.

(g. sulphated ash per 100 g. leather).

Treatment of leather.*	Grain (1.0 mm.)	Corium	Average
LEATHER FROM EXPERIMENT I—			
Saturated $MgSO_4$	12·5	8·2	9·1
Saturated $MgSO_4$ plus 50% cane sugar ...	7·0	6·1	6·3
Saturated $MgSO_4$ plus 50% glucose... ...	8·7	6·3	6·7
LEATHER FROM EXPERIMENT II—			
Control	—	—	1·8
Saturated $MgSO_4$	9·0	2·8	3·8
Saturated $MgSO_4$ plus 50% cane sugar ...	6·0	—	4·1
Saturated $MgSO_3$ plus 50% glucose† ...	—	—	3·1
5% $MgSO_4$	—	—	2·0

* Percentages refer to concentration in solution.
† Glucose content of this sample was 3.0%.

Conclusions.

The results obtained indicate that treatment with magnesium sulphate, cane sugar or glucose before drying out has certain effects on the properties of the leathers which are mainly attributable to the effect of these substances on the vapour pressure of the moisture in the leather.

The various treatments caused a marked reduction in the rate at which water was removed from the leather in the first stages of drying. The effect of the different treatments on the time required to dry to constant weight, however, was negligible, firstly because the treated samples have less water to loose, and secondly because the first rapid drying during which the rate of evaporation is mainly influenced by the effective vapour pressure of the water in the leather, is soon superseded by a period of slower drying during which such factors as the rate of diffusion of the moisture to the surface of the leather and the extent to which the water is held by the protein fibres have an appreciable effect.

One of the chief effects of treatment in magnesium sulphate and sugar solutions is to decrease the amount of water held by the leathers at the beginning of drying. In several cases this reduction was as much as 50%. The apparent dehydrating action of the salts and sugar solutions may be ascribed to the fact that the osmotic concentration of these solutions is greater than that of the tan solution present in the spaces in the leather, and water, therefore, tends to diffuse from the leather into the solution so as to equalise the two concentrations. All the leathers dried to approximately the same moisture content, so that any reduction in the initial water content caused a corresponding decrease in the amount of water lost during drying. In these experiments the leathers were immersed in solutions of magnesium sulphate and sugars and it does not

necessarily follow that drumming with the solid compounds will have a similar dehydrating action.

The smaller the amount of water that is lost during drying the less possbility will there be of water solubles creeping to the surface and hence treatment in magnesium sulphate and sugar solutions should decrease the likelihood of the occurrence of excess water solubles in the grain by reason of its partial dehydrating action as well as by precipitation of the tan.

The water contents of the leathers at high humidities were also affected by the different treatments. At 75% R.H. this effect was just appreciable, at 85 and 100% R.H. all the treated samples took up more moisture than the controls.

It would appear, therefore, that the presence of magnesium sulphate, cane sugar or glucose in leather causes it to take up more moisture, and hence liable to become soft when exposed to high humidities (greater than 85% R.H.). This effect is especially marked when magnesium sulphate and sugar are present together ; it is suggested that water is first absorbed by the sugar, and this causes the magnesium sulphate to go into solution, the vapour pressure of which is lower than that of the solid. At low humidities, however, the presence of magnesium sulphate and sugars does not cause the leather to retain moisture. The non-crackiness of American leathers under dry condition is probably not directly due to the comparatively large amounts of magnesium sulphate and glucose in these leathers, but to the effect of the salts and sugar treatment on the nature and amount of the water soluble material present in the grain layer. The magnesium sulphate causes precipitation of the tannins in the leather, thus reducing the amount of water soluble material of this nature in the grain, while the dehydrating action of both the magnesium sulphate and the sugar, by reducing the amount of water lost during drying, will diminish the possibility of water soluble material creeping to the surface.

The statement that leathers containing glucose are more liable to absorb moisture and become soft when exposed to moist conditions than leathers containing a corresponding amount of cane sugar appears to be only partially correct. In these experiments it has been found that at 85% R.H. and below, samples treated in cane sugar and glucose behaved similarly. It was only at 100% R.H. that those treated with glucose took up more moisture than the corresponding samples treated with cane sugar, and even then the differences were slight.

As would be expected, it was found that the moisture uptake of the samples was to a large extent dependent on the amount of magnesium sulphate and sugar present in the leather.

It was interesting to find that in the samples treated in magnesium sulphate alone the salt was mainly present in the grain layer, while in samples treated in solutions of magnesium sulphate and sugar the salt was more evenly distributed throughout the leather. This agrees with the suggestion put forward in an earlier paper[1], namely that one function of the sugar is to prevent the formation of clots of pecipitated tan near the surface of the leather, thus hindering further diffusion of the salt solution into the leather. This observation is also

of interest in connection with the formation of salt spue. If the magnesium sulphate were initially located mainly in the grain there would be more tendency for spue formation than if an equivalent amount were distributed throughout the leather.

Summary.

1. Immersion in solutions of magnesium sulphate and sugars has a dehydrating action on wet leather, in some cases reducing the water content by as much as 50%. The amount of water lost in drying to constant weight is thereby reduced, and it is suggested that this will reduce the tendency of water soluble material to creep to the surface during drying.

2. Treatment in salts and sugar solutions also decreases the rate at which water is lost in the initial stages of drying ; this is attributed to the influence of these materials on the vapour pressure of the water in the leather.

3. At high humidities (85% R.H. and higher) the presence of magnesium sulphate and sugars increases the water absorption of leathers, but at lower humidities has little effect.

4. In leathers treated with magnesium sulphate alone, the salt was mainly present in the grain layer, while in samples treated with magnesium sulphate and sugars the salt was more evenly distributed throughout the leather. This is probably a contributory cause of the prevention of salt spue by sugars.

Thanks are due to the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

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THE USE OF SALTS IN THE FINISHING OF SOLE BENDS.

Part IV.—The Effect of Immersion in Solutions of Magnesium Sulphate, Cane Sugar and Glucose on Some Physical Properties of the Finished Leather.

By J. H. Bowes, F. H. Quinn and C. L. Ward.

Various aspects of the use of magnesium sulphate and sugars in the finishing of sole bends have been discussed in earlier papers[1, 2, 3]. It has been shown that magnesium sulphate precipitates the water soluble material in the leather, and that although cane sugar and glucose decrease the amount of precipitate formed, their presence is probably beneficial in that they cause the precipitate to be more evenly distributed throughout the leather[1], and retard the formation of salt spue[2]. Treatment with magnesium sulphate and sugars has also been shown to decrease the amount and rate of water loss during drying[3], thereby reducing the tendency of water soluble material to creep to the surface during this process. The presence of magnesium sulphate and sugars in the leather increases water absorption at high humidities, but does not appreciably affect it at humidities below 50% R.H. It is suggested that the reported effect of salts and sugars in reducing the crackiness of leather is not directly due to their presence in the leather, but to their effect on the amount, nature and distribution of the uncombined material present in the leather.

Experiments have now been carried out on large pieces of leather to determine whether the conclusions reached as a result of laboratory experiments are substantiated in practice, and to determine the effect of treatment with magnesium sulphate and sugars on such physical properties as crackiness and flexibility which can only be properly studied on leather which has been given the normal finishing processes.

Experimental Method.

The tanning, treatment with magnesium sulphate and finishing of the samples was carried out at Puritan Tanneries, Ltd.

Pieces of fore-ends, approximately 18 in. × 12 in. and weighing between 2 and 2½ lbs. were cut from bends taken from the last tan liquors. The fore-ends were carefully chosen from bends of approximately the same substance and area in order to avoid as far as possible any difference in physical properties due to variation in the original samples.

The tannage was mainly catechol, a blend of eucalyptus, quebracho and mimosa with some valonia and myrabolam being used.

The samples were placed in shallow trays and covered with 4 litres of magnesium sulphate* and sugar solutions of various concentrations (see Table I). The addition of sodium alginate in the place of sugar was also tried.

* Quantities are expressed throughout as anhydrous magnesium sulphate, ($MgSO_4$). 1g. $MgSO_4$ is approximately equivalent to 2g. Epsom salts ($MgSO_4\ 7H_2O$).

TABLE I.

IMMERSION OF LEATHER IN SOLUTIONS OF MAGNESIUM SULPHATE AND SUGARS OF VARIOUS CONCENTRATIONS.

Solutions*	Barkometer Before Use	Barkometer After Use	Colour of solutions after use	Colour of finished leathers
Nil	—	—	—	Dark
5%$MgSO_4$	52	55	Brown	Fairly light
10%$MgSO_4$	104	106	Light brown	Light
Sat'd $MgSO_4$	242	232	Very light brown	Fairly light
10% $MgSO_4$+20% cane sugar ...	162	166	Light brown	Light
10% $MgSO_4$+20% glucose ...	145	154	Light brown	Light—rather grey
10% $MgSO_4$+0·5% sodium alginate	86	91	Brown	Fairly light
Sat'd $MgSO_4$+20% cane sugar	295	285	Very light brown	Light
Sat'd $MgSO_4$+20% glucose ...	292	285	Light brown	Light—rather grey
Sat'd $MgSO_4$+0·5% sodium alginate	210	210	Light brown	Fairly light
40% cane sugar	150	150	Brown	Fairly light
40% glucose	61	76	Dark brown	Fairly light
20% cane sugar	80	85	Brown	Rather dark
20% glucose	32	41	Dark brown	Dark

* Quantities are expressed throughout as anhydrous magnesium sulphate $MgSO_4$. 1g. $MgSO_4$ is equivalent to approximately 2g. Epsom salt $MgSO_4\ 7H_2O$.

After 20 hrs. the samples were removed, surface rinsed, drained, oiled on flesh and grain, dried, conditioned, set, re-oiled lightly, conditioned, rolled off and finally stoved for 24 hrs. at 83°F.

The barkometer strength of the solutions before and after use, the colour of the solutions after use, and the colour of the finished leathers, were recorded (Table I). The samples were tested for crack and flexibility after conditioning at 35, 70 and 96% R.H. (Table II). Sampling positions are shown in Fig. 1.

The crackiness was measured by the method described by Jordan Lloyd and Merry[4] and the same arbitrary scale for the degree of crack was used. Thus, a value of 1 means that the grain could not be cracked even when the leather was bent double on itself, a value of 2 that the leather did not crack round cylinder 0.5 ins. in diameter, but did crack when bent double on itself, and so on.

The flexibility was determined using an apparatus described by Burton[5]. Pieces of leather 5 in. by 2.5 in. were clamped along the two long edges so that there is a distance of 1 in. between the two clamps. The upper clamp is fixed in a stand and the lower clamp supports a pendulum of approximately 10 lbs. weight. The flexibility is expressed as the number of times the pendulum swings between two fixed points ; the larger the number of swings the greater is the flexibility.

The water absorption in a saturated atmosphere, Q_{15}, Q_{24}, the free water, the percentage loss on soaking and the apparent density before and after soaking were also measured (Table III). The water solubles in the grain (1 mm. split) and the corium were determined by the Official Method. The samples for extraction were cut into small pieces in the case of the grain and shaved in the

usual way in the case of the corium. The ash and sugar content of the water solubles were also determined. The water solubles content of the whole thickness was calculated from the values obtained for the two layers separately and a knowledge of the ratio of the weight of the grain to that of the corium. The results are given in Table IV.

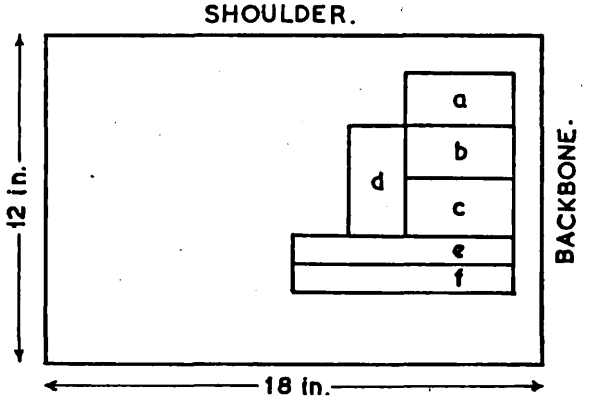

FIG. 1.

Flexibility Tests.
(*a*) Conditioned at 35% R.H.
(*b*) „ „ 70% R.H.
(*d*) „ „ 95% R.H.
Grain Crack Test.
(*e*) Conditioned at 35% R.H.
(*f*) „ „ 70% R.H.
Analysis.
(*c*) Conditioned at 70% R.H.

A figure designated " tannin " water solubles was also obtained by subtracting the sugar and salts content of the water solubles from the total water solubles. In calculating the salts content of the water solubles it was assumed that magnesium sulphate was the only salt present, and a correction was made for the water retained by this salt under the conditions of drying of the water solubles; this corresponded to approximately two molecules of water per molecule of magnesium sulphate.

Experimental Results.

(*a*) COLOUR OF SOLUTIONS.

The colour of the solutions indicated that little tan was dissolved out during the treatments except when cane sugar or glucose were used alone. As would be expected the amount of tan dissolved out decreased with increasing concentration of magnesium sulphate.

(*b*) APPEARANCE OF LEATHERS.

All the treated leathers were a better colour than the control. Those which had been immersed in solutions of magnesium sulphate and sugar were light in colour; those immersed in magnesium sulphate alone were fairly light in colour, while immersion in either cane sugar or glucose solutions alone produced a rather less good colour.

When stored under laboratory conditions no spue was observed on any of the leathers even after one year. On drying after exposure to an atmosphere of a 100% R.H. spue could be seen on several of the samples, notably those treated with saturated magnesium sulphate plus sodium alginate, 5% magnesium sulphate and saturated magnesium sulphate plus 20% glucose. The amount of spue on the remainder of the samples was negligible.

(*c*) Flexibility Tests.

The results of these tests are given in Table II. The figures recorded are the number of swings between two fixed points, the greater the number of swings the greater the flexibility of the leather. In spite of careful selection of the original fore-end samples there were considerable variations in thickness between the samples, which complicates the results and renders it difficult to draw any definite conclusions. The flexibility as measured by this method appears to be independent of the treatment which the leathers had received, and is not appreciably affected by changes in the relative humidity.

TABLE II.

The Effect of Immersion in Magnesium Sulphate and Sugar Solutions on the Crackiness and Flexibility of the Finished Leathers.

Treatment.	Approximate Thickness of Samples (mm)	Crack.		Flexibility.		
		R.H. 70%	R.H. 35%	R.H. 95%	R.H. 70%	R.H. 35%
Nil	4·4, 4·6	3	6+	15	16	17
5% $MgSO_4$	3·3, 3·5	2	5	28	29	28
10% $MgSO_4$	3·7, 4·0	2	1	19	16	19
Sat'd $MgSO_4$	4·2, 4·4	3	4	14	13	16
10% $MgSO_4$	3·8, 4·0	2	1	19	16	19
10% $MgSO_4$+20% cane sugar ...	4·2, 4·3	1	3	12	10	11
10% $MgSO_4$+20% glucose ...	4·3, 4·4	2	2	11	11	11
10% $MgSO_4$+0·5% sodium alginate	3·6, 3·8	2	2	21	19	23
Sat'd $MgSO_4$	4·2, 4·4	3	4	14	13	16
Sat'd $MgSO_4$+20% cane sugar ...	4·7, 4·8	3	3	8	7	9
Sat'd $MgSO_4$+20% glucose ...	5·0, 5·2	3	4	7	16	8
Sat'd $MgSO_4$+0·5% sodium alginate	3·9, 4·2	2	2	13	12	15
40% cane sugar	4·0, 4·1	3	6	12	14	13
40% glucose	3·7, 3·8	1	1	13	14	15
20% cane sugar	4·7, 4·9	2	5	7	9	8
20% glucose	4·0, 4·3	2	2	18	17	18

(*d*) Grain Crack.

Grain crack is less influenced by thickness than flexibility, and certain general conclusions may be drawn from the figures obtained.

The majority of the leathers were only slightly cracky at 70% R.H. and it was, therefore, decided not to test the leathers conditioned at 90% R.H. which would tend to be still less cracky.

At 70% R.H., the effect of immersion in the different solutions was hardly appreciable, but at 35% R.H. the effects were more definite. All the treated leathers cracked less readily than the control. Immersion in 10% magnesium sulphate either with or without the addition of cane sugar or glucose reduced

the crackiness to a greater extent than immersion in the corresponding saturated solutions. This may be due to the presence of a higher percentage of magnesium sulphate in the grain of the leathers immersed in the saturated solutions. The water soluble ash in the grain layer of the samples treated in saturated solutions was of the order of 7% whereas that of the samples treated in the 10% solutions was between 3 and 4%.

Immersion in cane sugar solutions caused no appreciable reduction in crackiness, whereas immersion in the corresponding glucose solutions definitely decreased the tendency to crack. When used in conjunction with magnesium sulphate, however, the two sugars behaved similarly.

(*e*) Apparent Density.

The values vary from 1.063 to 1.150 before soaking and from 0.829 to 0.949 after soaking. Leathers treated in saturated solutions of magnesium sulphate tend to have a high value for apparent density before soaking, but otherwise there appears to be no relationship between the values obtained and the treatment of the samples.

(*f*) Water Absorption.

The values of Q_{15} and Q_{24} for leather treated in saturated solutions of magnesium sulphate, either alone or with the addition of sugars, tend to be low, while those for leathers treated in glucose solutions alone tend to be high.

No relationship between the values of free water and the treatments which the samples received can be traced. The values of the majority of the samples lie between 35 and 39, leathers treated in 40%, glucose and saturated magnesium sulphate and cane sugar have high values (43.4 and 43.8) and the sample treated in saturated magnesium sulphate alone, a low value (30.2).

The percentage loss on soaking is high for samples treated in saturated solutions of magnesium sulphate with the additions of either cane sugar or glucose (27.9 and 23.8 respectively).

(*g*) Water Contents of the Leather at Different Relative Humidities.

At all humidities the samples treated with magnesium sulphate had a higher water content than that of the untreated sample; the higher the humidity the greater being this difference. The water content could not, however, be related to the magnesium sulphate and sugar content of the leathers. Samples treated with sugars only tended in many cases to have a slightly lower water content than that of corresponding samples treated without sugar.

Water Soluble Material.

With two exceptions, saturated magnesium sulphate and 10% magnesium sulphate plus alginate, the treatments increased the total water solubles in the leather, but caused it to be more evenly distributed. In the control the total water solubles in the grain were approximately double that in the corium whereas in the treated samples it was approximately equally distributed

TABLE III.

THE EFFECTS OF IMMERSION IN MAGNESIUM SULPHATE AND SUGAR SOLUTIONS. ON THE WATER ABSORPTION OF THE FINISHED LEATHERS.

Treatment	Thickness in mm.	Apparent Density		Q_{15}	Q_{24}	Free Water	% Loss on Soaking	Water content g. per 100g. dried leather		
		Before Soaking	After Soaking					60% R.H.	85% R.H.	100% R.H
Nil	4·4	1·076	0·905	12·8	23·8	37·8	14·0	12·0	15·6	30·5
5% $MgSO_4$	3·5	1·067	0·898	20·5	39·1	50·1	11·0	12·8	18·1	36·1
10% $MgSO_4$	3·8	1·078	0·876	19·5	26·3	39·4	13·1	14·7	22·2	42·7
Sat'd $MgSO_4$	4·4	1·117	0·925	10·7	12·9	30·2	17·3	14·3	20·3	33·3
10% $MgSO_4$	3·8	1·078	0·876	19·5	26·3	39·4	13·1	14·7	22·2	42·7
10% $MgSO_4$+20% cane sugar	4·5	1·097	0·892	15·2	19·5	36·3	16·8	11·7	19·4	39·2
10% $MgSO_4$+20% glucose	4·4	1·117	0·927	15·0	20·4	35·5	15·1	11·5	16·1	36·3
10% $MgSO_4$+0·5% sodium alginate	3·5	1·120	0·949	18·4	24·6	35·1	10·5	13·7	19·3	38·3
Sat'd $MgSO_4$	4·4	1·117	0·925	10·7	12·9	30·2	17·3	14·3	20·3	33·3
Sat'd $MgSO_4$+20% cane sugar	4·6	1·150	0·829	10·5	15·9	43·8	27·9	13·4	19·0	35·6
Sat'd $MgSO_4$+20% glucose	5·4	1·078	0·847	11·1	12·9	36·7	23·8	15·2	20·8	37·8
Sat'd $MgSO_4$+0·5% sodium alginate	3·8	1·129	0·919	13·5	15·8	35·4	19·6	14·2	20·9	40·3
40% cane sugar	4·5	1·104	0·901	11·4	19·0	35·2	16·2	10·1	15·3	32·9
40% glucose	3·5	1·063	0·873	21·8	30·0	43·4	13·4	8·4	13·4	28·1
20% cane sugar	4·7	1·097	0·924	13·1	22·1	34·2	12·1	11·9	16·2	32·4
20% glucose	4·1	1·131	0·916	17·0	29·6	37·2	7·6	11·0	15·9	27·3

between the grain and corium. Owing to this more even distribution, the total water solubles content of the grain of the treated samples was less than that of the control in spite of the fact that the amount present in the full thickness was greater.

The " tannin " water solubles in the full thickness of the leather were not greatly affected by the treatments, except in the case of saturated magnesium sulphate, but again there was a more even distribution between grain and corium. So that the amount in the grain of the treated samples was considerably less than that in the grain of the control. Since the precipitate formed by the addition of magnesium sulphate to tan liquors is largely soluble in water it would not be expected that treatment of leather in solutions of this salt would greatly decrease the " tannin " water solubles as determined by the official method.

The salts and sugar contents of the leathers was increased in accordance with the treatments given, examination of the results showing that the increase is approximately equivalent to the increase in total water solubles.

These results confirm the suggestions put forward in earlier papers[1, 3], namely that saturated magnesium sulphate solution alone does not readily penetrate into the leather because it causes precipitation of the tannin in clots which hinder further diffusion, the tannin water soluble material, therefore, creeps to the surface during drying and is precipitated by the magnesium sulphate in the grain. In the presence of sugar or with less concentrated solutions of magnesium sulphate, however, the salt diffuses throughout the leather, the tannin is precipitated in the corium as well as the grain and there is little or no migration of the water soluble material towards the surface during drying.

The Relationship Between the Water Solubles Content and the Physical Properties of the Leather.

There is no relationship between Q_{15}, Q_{24}, or free water, and the amount of water soluble material present, either total, tannin, or the sum of the magnesium sulphate and sugar.

The crackiness of the leathers at low humidities shows some relationship with the water soluble material content of the grain, especially the " tannin " water soluble material. Leathers having less than 20% " tannin " water solubles in the grain have figures for crack of 2 or less, and leathers containing a high percentage of tannin water soluble material in the grain have high figures.

Conclusions.

As already pointed out, there are obvious limitations to the conclusions which can be drawn from this investigation. In spite of careful selection of the original samples of leather the variation in physical properties due to differences in the original samples must be taken into consideration. In spite of these difficulties, however, certain conclusions may be reached. The most important of these is that treatment in solutions of magnesium sulphate, cane sugar, or glucose tends to reduce the crackiness of leather at low humidities.

TABLE IV.

WATER SOLUBLES CONTENTS.

(g. per 100 g. leather dried at 100°C.).

Treatment	Total Water Solubles.			Ash			Sugar.			Salts + Sugar			Tannin Water Solubles		
	Grain	Corium	Full Thickness	Grain	Corium	Full Thickness	Grain	Corium	Full Thickness	Grain	Corium	Full Thickness	Grain	Corium	Full Thickness
Control	37·42	17·98	21·25	1·82	1·65	1·69	0·28	0·19	0·20	2·64	2·33	2·40	34·78	15·65	18·85
Saturated $MgSO_4$	37·95	16·58	19·67	7·09	5·71	5·91	0·46	0·56	0·54	9·68	6·98	8·23	28·27	9·60	11·44
10% $MgSO_4$	23·17	25·45	25·08	4·06	4·65	4·54	0·55	0·70	0·68	5·83	6·75	6·58	17·34	18·70	18·50
5% $MgSO_4$	27·04	23·82	24·54	3·55	3·58	3·57	0·38	0·27	0·29	4·99	4·92	4·93	22·05	18·90	19·61
10% $MgSO_4$	23·17	25·45	25·08	4·06	4·65	4·51	0·55	0·70	0·68	5·83	6·75	6·58	17·34	18·70	18·50
10% $MgSO_4$ + 20% cane sugar	29·17	28·73	28·81	3·39	3·24	3·25	0·92*	1·47*	1·33*	5·33	5·68	5·56	23·84	23·05	23·25
10% $MgSO_4$ + 20% glucose ...	27·58	27·32	27·40	3·64	2·89	3·02	4·03	4·19	4·17	8·76	7·95	8·10	18·82	19·37	19·36
10% $MgSO_4$ + 0·5% sodium alginate	18·04	21·14	20·48	4·03	4·11	4·10	0·84	0·64	0·67	6·08	5·99	6·00	11·96	15·15	14·48
Saturated $MgSO_4$	37·95	16·58	19·67	7·09	5·71	5·91	0·46	0·56	0·54	9·68	6·98	8·23	28·27	9·60	11·44
Sat'd $MgSO_4$ + 20% cane sugar ...	35·74	36·19	36·14	10·73	6·58	7·18	2·97*	2·85*	2·89*	16·92	11·41	12·24	18·82	24·78	23·90
Sat'd $MgSO_4$ + 20% glucose ...	39·59	34·86	35·62	7·66	7·07	7·15	5·15	5·13	5·13	15·11	14·32	14·42	25·27	20·54	21·20
Sat'd $MgSO_4$ + 0·5% sodium alginate	28·97	27·70	28·03	7·31	4·96	5·41	1·88	1·62	1·68	11·38	8·07	8·72	17·59	19·63	16·90
40% cane sugar	39·00	27·36	30·28	1·28	3·28	3·00	3·54*	1·34*	1·63	5·05	5·61	5·54	33·66	21·77	24·75
40% glucose	23·25	23·59	23·56	0·94	1·19	1·15	3·71	4·60	4·43	4·94	6·15	5·93	19·31	17·44	17·63
20% cane sugar	25·08	26·58	26·36	1·03	0·94	0·95	1·77*	1·89*	1·81*	3·21	3·12	3·15	21·87	23·46	23·31
20% glucose	25·23	21·19	21·90	1·30	1·21	1·22	2·31	2·48	2·45	4·01	4·06	4·04	21·22	17·13	17·86

* Expressed as cane sugar, all other values expressed as per cent. glucose.

Glucose was definitely more effective than cane sugar in this respect and treatment in 10% magnesium sulphate more effective than treatment in a saturated solution.

This reduction in crackiness at low humidities appears to be associated with the effect of the salts and sugar treatment on the distribution of water soluble material in the leather. The presence of a large amount of water soluble material originating from the tan liquor has been found to be associated with cracky grain[6], probably owing to the fact that it tends to dry out as a continuous brittle film. Immersion in magnesium sulphate and sugar solutions tends to reduce the amount of "tannin" water soluble material in the grain layer, by precipitating the tannins in the leather, and thus reducing the tendency of this to creep to the surface during drying. The precipitation of the tannin in the form of discrete particles so preventing the formation of a continuous brittle film of dried tan is probably a further factor influencing crackiness.

It might be expected that treatment in concentrated sugar and salt solutions would tend to increase the water absorption and the percentage loss on soaking owing to the high proportion of magnesium sulphate and sugars, which these leathers must contain (see Table IV). This, however, is not necessarily so ; in fact, leathers treated in saturated solutions of magnesium sulphate tend to have low values for Q_{15}, and Q_{24}, and only those leathers treated with saturated magnesium sulphate and cane sugar or glucose give values higher than the average for percentage loss on soaking. The moisture content of leathers treated with magnesium sulphate, however, were higher than that of the control, especially at high humidities.

In general, therefore, it may be stated that with this particular type of rather astringent tannage, treatment in magnesium sulphate and cane sugar or glucose decreases the tendency of leathers to crack at low humidities, and increases the absorption of moisture at high relative humidities, but in general does not appreciably effect the flexibility, water absorption (Q_{15} and Q_{24}), free water or percentage loss on soaking except in a few isolated instances which appear to bear no relationship to the concentration of sugar and salts used.

Summary.

1. Pieces of fore-end were taken from the last tan liquors, immersed in solutions of magnesium sulphate and sugars and finished as sole leather in the normal way.

2. In all cases the treatments improved the colour of the leather ; after storage for a year at varying humidities only those samples treated in saturated magnesium sulphate plus 0.5% sodium alginate or 20% glucose showed any appreciable salt spue.

3. The flexibility of the leather was not affected by the treatments or by changes in humidity between 35 and 95% R.H. At low humidities the treated leathers cracked less readily than the control. Treatment in 10% magnesium sulphate with or without the addition of sugar reduced crackiness to a great extent than treatment in the corresponding saturated solutions.

4. There was no obvious relationship between the treatments given and the values for Q_{15}, Q_{24}, percentage loss on soaking or free water. The leathers treated with saturated and 10% solutions of magnesium sulphate absorbed more water than the control especially at high humidities.

5. All the treatments, with the exception of that in saturated magnesium sulphate alone, increased the total water solubles but caused a more even distribution between the grain and corium. In contrast treatment in saturated magnesium sulphate alone did not prevent the accumulation of water solubles in the grain. It is suggested that the magnesium sulphate does not penetrate the leather because it causes precipitation of the tannin in clots which hinder further diffusion, some of the water soluble material, therefore creeps towards the surface during drying and is only precipitated by the magnesium sulphate when it reaches the grain.

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1–6, *Nelson Square, London, S.E.*1.

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A NOTE ON THE CALCULATION OF HIDE SUBSTANCE FROM NITROGEN DETERMINATIONS.

By J. H. Bowes and R. H. Kenten.

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A NOTE ON THE CALCULATION OF HIDE SUBSTANCE FROM NITROGEN DETERMINATIONS.

By J. H. Bowes and R. H. Kenten.

In the course of investigations on the effect of liming on collagen, a number of values have been obtained for the total nitrogen content of limed pelt from various sources (see Table I) which indicate that in many cases the factor 5.62[1] used in the calculation of hide substance, and hence, of degree of tannage is not precisely correct.

The present figures for total nitrogen, with one exception, were determined by the method of Chibnall, Rees and Williams[2]. These workers recommend that heating should be continued for 8 hrs. after the solution has cleared owing to the resistance of certain peptides to hydrolysis ; this, however, does not appear to be necessary with collagen (*c.f.* results on hide powder Table I).

TABLE I.

NITROGEN CONTENTS OF SOME LIMED COLLAGENS.

Source of Collagen.	Total Nitrogen Moisture—and ash-free collagen.
Hide Powder Batch C.10	18·0
Hide Powder Batch C.12* ... ... (digested until no yellow hue—approx. 2 hrs. after all carbon gone)	18·0
Ox-hide Corium—no chemical treatment	18·6
Oxhide—pit-limed 8 days with 0·06–0·09% Na_2S in liquor	18·3
„ „ 7 „ 0·03–0·09% „ „	18·1
„ „ 8 „ 0·04–0·08% „ „	18·1
„—drum-limed 3 „ 0·8% Na_2S in liquor	18·1
Calf-skin—painted and limed 4 days	18·2
„ „ „ 4 „	18·2
„ limed 7 days	18·0
Sheepskins—painted, washed, limed 7 days and pickled in H_2SO_4 and NaCl 1	17·2
2	17·1
3	17·2
4	17·4
5	17·3

* Determination made by D. Fowler.

Assuming that the nitrogen content of limed ox-hide collagen is 18.1%, and not 17.7% as is presupposed in the use of the factor 5.62, the error involved in calculating the degree of tannage varies from 3% when the degree of tannage is 80 to nearly 20% when the degree of tannage is 10 (see Table II). With sheepskins of lower nitrogen content (factor 5.81) the error is larger, and is in the reverse direction (Table II, col. 5).

TABLE II.

CALCULATION OF DEGREE OF TANNAGE USING ALTERNATIVE FACTORS FOR CONVERSION OF NITROGEN TO HIDE SUBSTANCE.

	Heavy Leather		Light Leather			Leather of very low degree of Tannage	
	Using 5·62 factor	Using 5·52* factor	Using 5·62 factor	Using 5·52* factor	Using 5·81† factor	Using 5·62 factor	Using 5·52* factor
g. per 100 *g. air dry leather.*							
Hide substance + Fixed Tan	63	63	70	70	70	77	77
Nitrogen	6·23	6·23	8·91	8·91	8·91	12·46	12·46
Hide Substance (calculated from nitrogen)... ...	35·0	34·4	50·0	49·1	51·8	70·0	68·8
Fixed Tan (by difference)	28·0	28·6	20·0	20·9	18·2	7·0	8·2
Degree of Tannage ...	80·0	83·2	40·0	42·6	35·1	10·0	11·9

*Calculated from nitrogen content of limed ox-hide, 18·1% (Table I).
†Calculated from nitrogen content of limed sheepskin, 17·2% (Table I).

In the analysis of heavy leather the error is small, and since the exact nitrogen content of the original limed pelt cannot be determined, there would appear to be little object in changing to a new factor, which though possibly more accurate in itself, would not allow of ready comparison with established figures.

With sheepskins, the error may be more serious, but again since the exact nitrogen content of the pelt prior to tannage cannot be determined, the advisability of using a factor corresponding to a lower nitrogen content is doubtful.

In carrying out experimental work on the fixation of tanning by hide powder or skin, however, it is recommended that the nitrogen content of the original material be determined, and the appropriate factor used for the calculation of hide substance..

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British Leather Manufacturers' Research Association,
1–6, *Nelson Square, S.E.*1.

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THE YELLOWING OF PICKLED SHEEPSKINS.

By J. H. Bowes and G. O. Morse.

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THE YELLOWING OF PICKLED SHEEPSKINS.

By J. H. Bowes and G. O. Morse.

Pickled sheepskins are liable to become discoloured, especially when stored for long periods. This discolouration which varies from fawn to yellow, is found with both imported and domestic pickled skins.

The examination of a number of such skins, and comparison of the results with those obtained with New Zealand pickled skins from a similar source but free from discolouration (Table I), suggested that the discolouration might

be connected either with degradation of the collagen as indicated by the higher soluble nitrogen of the discoloured skins, or with oxidation of the grease as indicated by the consistently lower iodine value of the greases extracted from these skins. These values were definitely lower than the average values usually obtained with this type of skin.

TABLE I.

SOME ANALYSES OF NORMAL AND DISCOLOURED NEW ZEALAND PICKLED SHEEPSKINS.

	Per 100 g. Collagen				Analysis of Grease			
pH of aqueous extract	Soluble nitrogen (g.)	Total acid (*ml N*)	Sodium chloride (g.)	Iron (mg.)	Grease (% in extracted skin)	Iodine value	% Free fatty acid (as oleic acid)	Iron mg. per 100 *g.* grease
Discoloured skins								
2·32	0·255	14·28	35·9	0·87	30·9	46·1	15·8	16·4
2·25	0·289	18·02	41·9	—	23·8	33·6	9·4	—
2·12	0·318	14·64	26·2	1·74	11·3	37·5	4·6	—
Skins free from discolouration								
2·40	0·171	13·34	32·3	—	6·1	70·6	10·4	—
2·46	0·185	13·98	24·4	—	1·6	77·8	9·0	—
2·46	0·228	18·21	35·3	—	43·6	55·5	11·6	15·6

In an earlier investigation on the effect of storage in the pickled and salted condition on the grease, Balfe, Bowes, Innes and Pleass[1] concluded that there was no evidence that oxidation of the grease occurred. Assuming that the iodine values of skin grease lie between 35 and 50, they found no indication of any change during storage and considered it improbable that significant oxidation can take place owing to the comparatively small amount of unsaturated fatty acids in the grease. However, the iodine values of the normal skins (Table I) and of other freshly pickled sheepskins indicate that the iodine value of the grease in fresh sheepskins must be of the order of 58 to 70, or even higher in some cases. Koppenhoefer[2] has obtained similar values (68.6, 64.4 and 57.5) for the corium grease of sheepskins. The findings of Balfe, *et al*, do not, therefore exclude the possibility of oxidation of the grease under certain circumstances.

In connection with degradation of the collagen, Pleass[3] has observed that pelt pickled in high concentration of acid and stored at 37°C tends to go brown and mushy ; this is probably an extreme case of the yellowing of pickled sheepskins in practice. There is some indication in the figures given in Table I, that the discoloured pelts may have been pickled in higher concentrations of acid than the normal pelts.

In the present investigation, experiments have been carried out under controlled conditions to determine whether oxidation of the grease and degradation of the collagen are in fact the causes of yellowing, and to determine the conditions of pickling and storage which are likely to produce the changes leading to yellowing.

Experimental Method and Results.

The experiments covered the storage at different temperatures of skins which had undergone various pretreatments. These included pickling in different concentrations of acid, the addition of substances likely to retard or accelerate the oxidation of the grease, and the removal of the natural grease before pickling. All pickling was carried out in liquors containing sulphuric acid and sodium chloride. After pickling the samples were wrapped in oiled silk to prevent drying and stored in stoppered bottles at temperatures of 0°, 20°, 30° and 37°C for 6 months. The grease was then extracted from the samples with acetone in a Soxhlet extractor. The grease was separated from the aqueous acetone with petroleum ether and the final extract dried over calcium chloride, evaporated and then dried for 1 hour at 100°C. The percentage grease, the iodine value and the free fatty acid content were recorded. The oxidised fatty acids were not determined, since in a previous investigation[1] it was found that the values obtained were small and tended to vary with the unsaponifiable matter, due it was considered, to some of the fatty acids of the sterol ester greases being returned as oxidised fatty acids by the analytical method generally used. The total and soluble nitrogen of the skin were also determined. For the determination of the soluble nitrogen about 10 g. of air-dried skin were taken and extracted with 500 ml. of 0.1N $NaHCO_3$ solution for 24 hr. with intermittent shaking. 100 ml. of this extract were taken for the nitrogen determination by the usual Kjeldahl method. The pH values of the aqueous extracts of the pelts were determined when the size of the sample permitted.

(i) The Effect of Variations in Pickling and Storage on the Condition of the Pickled Skins.

The first experiment was designed to determine the effect of various conditions of pickling and storage on the oxidation of the grease and degradation of the collagen. Since there is a possibility that the composition of the grease in sheepskins may vary according to the position of the skin* and the pretreatment of the skin, it was considered advisable to degrease the samples and to replace the natural grease with a grease of known and constant composition so that a large number of comparable samples could be obtained. For this purpose samples of limed sheepskin were delimed in ammonium chloride, washed and degreased in several changes of acetone. The grease content of the samples after this treatment was less than 4%. A mixture of equal proportions of tallow and neatsfoot oil, dissolved in carbon tetrachloride, was added evenly to the flesh surface of the samples. Several additions were made the solvent being allowed to evaporate off between each, until the grease content of the samples approximated to 25 % on the dry weight of the skin. The samples were then pickled as indicated in Table II and stored at different temperatures for 6 months.

The chief factor affecting the colour of the samples, which varied from cream to dirty brown, was the temperature of storage, the higher the tempera-

* See Appendix

ture, the greater being the discolouration. The concentration of the acid in the pickle liquor affected the colour and condition of the samples to a lesser extent. Only those samples pickled with 1% sulphuric and stored at 0°C were unaltered in colour, and the samples stored at 37°C had definitely deteriorated and were soft, flabby and brown.

The pH values of the aqueous extract increased during storage, especially at high temperatures. For some reason the pH values of the samples pickled in the straight pickle liquor and stored at 20° were particularly high. The iodine values and the free fatty acid contents of the greases extracted from these samples were also anomalous. There was no corresponding increase in the amount of soluble nitrogen and it is possible that these samples were infected by some type of mould.

The soluble nitrogen expressed as a percentage of the total nitrogen increased with the temperature of storage in each series. The increase up to 20° was small, but at 37° the soluble nitrogen had increased up to 50% or more of the total nitrogen. In general, the values were higher in the series pickled in the high concentration of acid, especially when stored at 37°C.

The figures suggest that at the higher temperatures the presence of iron increases the formation of soluble nitrogen, while the presence of pyrogallol tends to retard its formation.

Both the colour of the extracted greases and their iodine values indicate that some oxidation of the grease occurred in the samples pickled in the straight pickle liquor and stored at 20° or 37°C. The concentration of the acid and the presence of ferric iron appeared to have no appreciable effect on the extent of this oxidation. Since the samples stored at 20° were apparently anomalous for some reason, there are insufficient results to enable any definite conclusion to be reached on this point. The iodine values of the greases were influenced by the presence of the pyrogallol but as there were no alterations in the values during storage, it may be inferred that pyrogallol inhibited any oxidation.

The free fatty acid content of the grease increased during storage in most cases. The increase was small in the samples stored at 0°, but at 20° and 37°C the increase was 50% or more in several instances. There is some indication that the presence of pyrogallol retarded the formation of free fatty acid.

(ii) The Effect of Temperature of Storage on the Oxidation of Grease.

In a second experiment the effect of temperature of storage on the natural grease of the skin was considered. In this case, in order to minimise variations in the initial composition of the grease, due to the location in the skin, small samples only were used, all taken from an area 6 in. by 8 in., symmetrically placed with respect to the backbone and 3 in. from the tail.

The results (Table III) suggest that the natural grease in the skin is less readily affected by conditions of storage than the grease mixture used in the earlier experiments. After 6 months' storage only the samples stored at 0° and 20° were still a good colour, while those stored at 30° and 37° were definitely discoloured.

TABLE II.

THE EFFECT OF VARIATION IN PICKLING AND STORAGE ON THE CONDITION OF THE PICKLED SKINS.

Conditions of Pickling and Storage	Colour of Sample	Colour of Grease	Sodium chloride (% of collagen)	pH of aqueous extract	Water Soluble Nitrogen (% of air-dry skin)	Total Nitrogen (% of air-dry skin)	Soluble Nitrogen (% Total N)	Grease (% of degreased skin)	Iodine value	Free fatty acid (% oleic acid on grease)
Pickled in 1% Sulphuric Acid										
Control—immediately after pickling	Normal	Light yellow	21·0	1·95	0·119	13·66	0·91	8·4	48·5	11·5
Stored at 0°C	Normal	Light yellow	—	2·14	0·147	9·67	1·52	22·4	49·7	12·8
20°C	Light brown patches	Brown	—	2·83	0·433	9·73	4·45	21·1	28·8	42·1
37°C	Light brown	Light brown	—	2·52	5·42	10·55	51·40	17·4	33·8	35·4
Ferric Alum ≡ 2% Fe added to pickle liquor										
Stored at 0°C	Normal	Light yellow	—	2·10	0·209	10·52	1·99	16·1	47·1	18·2
20°C	Fawn	Brown	—	2·33	0·565	9·02	6·27	26·3	39·8	22·4
0·5% *Pyrogallol added to pickle liquor*										
Control—immediately after pickling	Grey	Brown	21·0	1·95	0·119	13·66	0·91	8·4	48·5	11·5
Stored at 0°C	↓	↓	—	1·95	0·174	9·62	1·81	21·3	52·5	15·9
20°C			—	2·42	0·267	10·27	2·60	8·4	52·1	23·5
37°C	Dark grey	Dark brown	—	2·55	3·94	9·05	43·52	18·9	53·1	10·2
Pickled in 2% Sulphuric Acid										
Control—immediately after pickling	Normal	Light yellow	21·0	1·72	0·141	11·68	0·82	25·0	52·0	8·3
Stored at 0°C	Slightly yellow	Light yellow	—	2·16	0·273	10·07	2·71	20·8	49·2	16·4
20°C	Brownish yellow	Light brown	—	2·71	0·347	10·15	3·42	13·5	19·5	61·2
37°C	Brown	Dark brown	—	2·49	7·27	9·72	74·80	11·2	36·3	36·8
Ferric Alum ≡ 2% Fe added to pickle liquor										
Stored at 0°C	Yellow	Light brown	—	1·84	0·251	9·79	2·56	15·6	47·5	16·5
20°C	Fawn	Dark brown	—	2·07	0·895	11·44	7·82	25·2	39·4	24·2
0·5% *Pyrogallol added to pickle liquor*										
Control—immediately after pickling	Grey	Brown	21·0	1·73	0·141	11·68	0·83	18·3	61·6	16·7
Stored at 0°C	↓	↓	—	1·84	0·309	9·75	3·17	21·0	54·7	14·4
20°C			—	2·38	0·289	10·21	2·83	14·4	51·8	8·7
37°C	Dark grey	Dark Brown	—	2·46	6·35	11·20	56·8	14·9	53·6	13·8

With the exception of the sample stored at 37°C the iodine value of the extracted grease was hardly affected by storage. The grease extracted from this sample was also darker in colour than the other greases. The free fatty acid content of the grease was unaffected by storage at low temperatures, but at 30° and 37° there was some increase.

TABLE III.

THE EFFECT OF TEMPERATURE OF STORAGE ON THE IODINE VALUE AND FREE FATTY ACID CONTENT OF THE GREASE.

Conditions of Storage	Colour of Pelt	Grease % of degreased pelt	Iodine value	Free fatty acid as % oleic acid	Colour of Grease
Immediately after pickling	Normal	28·7	58·0	9·8	Light yellow
Stored at 0°	Normal	21·5	60·0	7·7	Light yellow
20°	Slightly yellow	18·3	58·8	8·9	Yellow
30°	Light yellow	22·4	59·8	11·0	Yellow
37°	Yellow	26·8	52·0	15·7	Dark brown
Addition of Antioxidants to Pickle Solution					
Immediately after pickling	Normal	—	68·5	19·6	Light yellow
No addition					
Stored at 0°	Normal	—	73·5	18·3	Light brown
20°	Slightly pink	—	78·0	12·0	Brown
30°	Light brown	—	80·0	21·8	Yellow
37°	Brown	—	73·2	18·2	Dark brown
0·1 *Cresol*					
Stored at 0°	Slightly yellow	—	88·7	17·6	Light brown
20°	Slightly pink	—	76·4	21·8	Brown
30°	Light brown		79·8	10·6	Light brown
37°	Brown	—	74·6	19·1	Dark brown
0·1% *p–nitrophenol*					
Stored at 0°	Normal	—	73·0	27·9	Dark brown
20°	Pinkish Brown	—	74·2	12·8	Dark brown
30°	Brown	—	65·1	32·4	Light yellow
37°	Brown	—	72·7	30·2	Dark brown

Initial pH of aqueous extract of pelt, 2·18. Sodium chloride content, 34·5 g. per 100 g. collagen.

The effect of the addition to the pickle liquor of substances which have been found to prevent the oxidation of the grease[4] was also investigated. A preliminary experiment was carried out in which 1% of each of the following pyrogallol, hydroquinone, β–napthol, α–napthylamine, *p*–nitrophenol and a mixture of *o*–, *m*– and *p*–cresols, were added to samples of the grease used in the first experiment. The samples were placed in an incubator at 30°C. After two weeks the samples to which additions had been made were all darker in colour than the control, due to the oxidation of the substances themselves, and only *p*–nitrophenol and the mixture of the cresols, which only caused slight darkening, were considered to be worth a further trial Accordingly, 0.1% of these two substances were added to pickle liquors in this experiment. There was again no evidence that the iodine value or the free fatty acid content of the grease was affected by the conditions of storage. (Table III).

(iii) THE EFFECT OF CONCENTRATION OF ACID IN THE PICKLE ON THE DEGRADATION OF THE COLLAGEN DURING STORAGE.

In a third experiment the natural grease was removed from the skin before pickling so that any yellowing due to oxidation of the grease was avoided and the effect of degradation of the collagen could be determined. The samples were then pickled in liquors containing varying amounts of sulphuric acid (Table IV).

TABLE IV.

THE EFFECT OF THE CONCENTRATION OF ACID IN THE PICKLE AND THE TEMPERATURE ON THE DEGRADATION OF THE COLLAGEN.

Conditions of Pickling and Storage	Colour of Skin	Soluble Nitrogen (% air-dry skin)	Total Nitrogen (% air-dry skin)	Water Soluble Nitrogen (% Total N_2)
0·45 *per cent.* H_2SO_4				
Stored at 0°C	Normal	0·20	11·94	1·64
20°C	Slightly yellow	0·63	10·68	5·90
30°C	Yellow	1·85	11·12	16·64
0.89 *per cent.* H_2SO_4				
Stored at 0°C	Normal	0·28	7·02	3·99
20°C	Slightly yellow	0·60	10·62	5·67
30°C	Yellow	1·86	11·32	16·41
1·43 *per cent.* H_2SO_4				
Stored at 0°	Normal	0·22	10·00	2·24
20°	Yellow	0·69	10·14	6·77
30°C	Deep yellow	2·32	10·84	21·40
1·86 *per cent* H_2SO_4				
Stored at 0°C	Slight yellow	0·22	7·72	2·91
20°C	Deep yellow	0·64	10·62	6·07
30°C	Deep yellow	2·09	10·50	19·91

Acid concentrations are expressed as a percentage of the pickle liquor after use.
Concentration of sodium chloride in the liquor = 16 per cent.

The colour of the skins was again mainly influenced by the temperature of storage and to a lesser extent by the concentration of acid in the pickle liquor. The soluble nitrogen also increased with temperature of storage but was not appreciably affected by the acid concentration. The colour of the sodium carbonate extracts of the discoloured pelts were correspondingly dark in colour and it is possible that the extracted substance which causes this colour is also responsible for the discolouration of the skin.

Conclusions.

The results as a whole indicate that the yellowing of pickled sheepskins is chiefly connected with the production of soluble nitrogen, *i.e.*, with degradation of the collagen, for both the soluble nitrogen and the yellowing of the skin are increased by the same conditions of pickling and storage. The first experiment suggested that oxidation of the grease was also connected with the discolouration, but subsequent experiments showed that oxidation of the natural grease does not readily take place during storage and further that degreased

skins yellowed as readily as undegreased skins. Hence, degradation of the collagen is probably the chief, if not the only, cause of yellowing.

It would appear, therefore, that yellowing is an indication that some deterioration of the skin has taken place and must be regarded as undesirable in all circumstances and not only when the skins are to be tanned for white leather.

Consideration of the results in Table I (normal skins) and Table V in Appendix A indicate that the greases extracted from New Zealand pickled sheepskins have rather higher iodine values (60 to 70) than the greases extracted from Welsh sheepskins (50 to 60) and hence might be more readily oxidised. This is the probable explanation of the low iodine values obtained in the first instance (Table I), oxidation of the grease having taken place either before pickling or during storage.

With regard to the conditions of pickling and storage which influence yellowing, the temperature of storage, and to a lesser extent the concentration of the acid in the pickle liquor, were found to be the most important factors. The results indicate that in order to minimise the chances of yellowing, pickled skins should be stored at as low a temperature as possible. In practice, however, the extent of yellowing and the increase in the soluble nitrogen is not very great unless the temperature of storage is 30°C or higher. The final concentration of acid in the pickle, while being high enough to avoid any possibility of mould growth or putrefaction, should not exceed about 1.5%. At higher concentrations there is a danger that the degradation of the collagen may be increased to an undesirable extent, especially if the temperature of storage is likely to be high.

TABLE V.

IODINE VALUES AND FREE FATTY ACID CONTENTS OF GREASES EXTRACTED FROM WELSH AND NEW ZEALAND PICKLED SHEEPSKINS.

Sampling Position (Fig. 1)	Grease (% of degreased skin)	Iodine value	Free fatty acid in grease (as % oleic acid)
Freshly pickled Welsh sheepskin			
1	11·9	60·1	—
3	19·6	58·2	—
5	28·7	58·1	—
6	11·0	58·2	9·8
Pickled Welsh sheepskin, stored 1 year—yellow			
1	25·1	54·8	23·4
3	11·8	54·1	26·3
5	22·0	54·9	18·5
New Zealand pickled sheepskin			
1	32·7	71·5	—
2	25·7	62·9	—
3	26·7	64·5	—
4	16·0	72·6	—
5	53·8	53·5	—
6	31·6	67·0	

Appendix A.

Variations in Composition of the Grease in Different Skins and Different Parts of the Skin.

In view of the fact that the variation in the composition of the grease from skin to skin and in different areas of the skin is often a subject of speculation, it was considered that some further results obtained in the course of this work might be of interest and were worth putting on record (Fig. 1 and Table V).

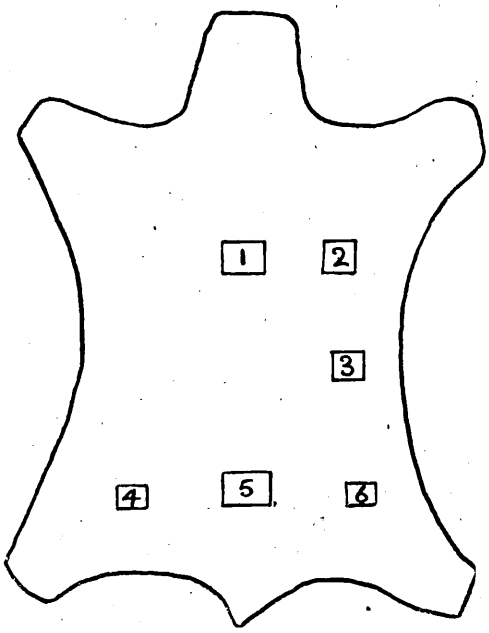

FIG. 1.
Location of samples taken for analysis of grease.

There was no variation in the iodine value of greases extracted from various parts of the Welsh skins, but a fairly wide variation in the case of the New Zealand pickled skin. The iodine value of the grease extracted from this and other New Zealand skins (see Table I—normal) are definitely higher than that of the Welsh skins, and other skins examined from time to time also conform to this finding.

Summary.

The examination of a number of pickled sheepskins which had become yellow during storage suggested that this discolouration was due either to oxidation of the grease or to degradation of the collagen.

The effect of various conditions of pickling and storage on the colour of the skins, the composition of the grease and the degree of degradation of the collagen has, therefore been studied.

The results indicate that yellowing is mainly due to degradation of the collagen, although oxidation of the grease may be a contributory cause in some cases. Storage at high temperatures was found to be the chief cause of degradation of the collagen and discolouration of the skins and it is suggested that during storage the temperature should not exceed 30°C (86°F). High concentrations of acid in the pickle also tend to increase the degradation of the collagen. There was no evidence that the natural grease of the skin was oxidised to any appreciable extent during storage.

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1. Balfe, Bowes, Innes and Pleass, *J.I.S.L.T.C.*, 1940, **24**, 329.
2. Koppenhoefer, *J.A.L.C.A.*, 1938, **33**, 203.
3. Pleass, *J.I.S.L.T.C.*, 1934, **18**, 464.
4. Bailey, " *Retardation of Chemical Reactions,*" 1937, p. 179

LEATHER

BY

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Collagen.

The electron microscope has been used by C. E. Hall, M. A. Jakus, and F. O. Schmitt to obtain further information on the molecular structure of collagen.[1] Collagen fibres obtained from a number of sources have been examined; these appear characteristically cross-striated with alternate opaque and transparent bands extending uniformly across the fibre. The average distance between these bands varies from 902 to 522 A., although the variations in any single preparation are more restricted. The authors consider that these striations arise as a consequence of the structure and arrangement of the collagen molecules in the fibre and are related to the same cause as the long-chain *X*-ray spacing in collagen. R. S. Bear,[2] working in the same laboratories, has recently reported this distance to be of the order of 640 A. It seems possible that these distances represent the length of the collagen molecule in the fibre.

The titration curves of collagen, hair, and silk fibroin have been determined by E. R. Theis and T. F. Jacoby,[3] using a new technique introduced by G. D. McLaughlin and R. S. Adams.[4] After exposure to solutions of varying pH values, the excess of acid or alkali is removed from the protein by pressure (7000 lb. per sq. in.) and the remaining combined acid or alkali determined directly by iodide–iodate–thiosulphate titration[5]; a correction is made for any hydrogen or hydroxyl ions remaining in the moisture still held by the protein after pressing. The titration curves are of the same general shape as those determined by other methods and are in accordance with the basic and acidic amino-acid content of the proteins. Definite maximum combining values are obtained at pH values of 1 and 13 corresponding with the fixation of 0·87 milliequivalent of acid and 0·43 milliequivalent of base respectively per 100 g. of collagen. The value for the maximum base-binding capacity is lower than that obtained by most workers[6] and is more in accord with the present analytical data

[1] *J. Amer. Chem. Soc.*, 1942, **64**, 1234; A., 1942, I, 297.
[2] *Ibid.*, 727; A., 1942, I, 231.
[3] *J. Amer. Leather Chem. Assoc.*, 1941, **36**, 545; B., 1942, II, 39.
[4] *Ibid.*, 1940, **35**, 44; B., 1940, 383.
[5] E. R. Theis and T. F. Jacoby, *ibid.*, 1941, **36**, 260; B., 1941, II, 362.
[6] C. L. A. Schmidt, "The Chemistry of the Amino Acids and Proteins," 1938, p. 343.

regarding the dicarboxylic acid content of collagen. In agreement with previous work, Theis and Jacoby find that the broad isoelectric zone is reduced by the addition of neutral salts.[7] In presence of salts the titration curves rise more steeply but the maximum amounts of acid and base combined are not affected. Recently J. Steinhardt and M. Harris[8] have put forward a new interpretation of this effect. They consider that the dependence of acid bound on the anion concentration, and of base bound on the cation concentration, can be explained on the assumption that anion and cation are also fixed stoicheiometrically by the protein. Wide differences in the shape of the titration curves obtained with different acids are ascribed to variations in the affinity of the anion of the acid for wool.[9] The extension of this work to collagen would be of interest since it offers a possible explanation of the effect of different salts in the tanning processes.

The glycine and proline content of gelatin determined by a new method has been found to be 26·0—27·0 and 17·1—17·8% respectively.[10] This method is based on the measurement of the extent to which the amino-acid present in the protein hydrolysate suppresses the solubility of one of its pure salts. Its application to protein analysis in general has recently been reviewed.[11]

The carbohydrate content of hide, bone, and tendon collagen has been determined by colorimetric methods and found to be equivalent to 0·029 millimol. of hexose sugar per gram of dry collagen.[12]

Preservation of Hides and Skins.

Several papers have appeared dealing with the curing and storage of hides and skins. R. W. Frey and L. S. Stuart[13] have found that well cured calf skins can be stored at low temperatures (2—3° c.) for 5 years without any significant deterioration in the chemical or physical properties. Immersion in brine solution prior to salting is reported to produce a leather with a stronger grain and better fibre structure, and the addition of zinc chloride or sodium hypochlorite to the brine to improve the quality of the leather.[14] Experiments with Frigorifico hides, however, indicate that brining before salting is of no advantage. The addition of 1% of sodium silicofluoride to the brine retarded deterioration when the

[7] D. Jordan Lloyd and A. Shore, "Chemistry of the Proteins," 1938, p. 329. J. B. Speakman and H. C. Hirst, *Trans. Faraday Soc.*, 1933, **29**, 148; A., 1933, 227. W. R. Atkin, *Stiasnyfestschrift*, 1937, 13; B., 1938, 303.

[8] *J. Res.Nat. Bur. Stand.*, 1940, **24**, 335, 519; B., 1940, 660; 1941, II, 77.

[9] J. Steinhardt, C. H. Fugitt, and M. Harris, *ibid.*, 1941, **26**, 293; B., 1941, II, 338. J. Steinhardt, *ibid.*, 1942, **28**, 191, 201; A., 1942, I, 238.

[10] M. Bergmann and W. H. Stein, *J. Biol. Chem.*, 1939, **128**, 217; A., 1939, II, 236.

[11] S. Moore, W. H. Stein, and M. Bergmann, *Chem. Rev.*, 1942, **30**, 423.

[12] J. Beek, jun., *J. Amer. Leather Chem. Assoc.*, 1941, **36**, 696; B., 1942, II, 206.

[13] *Ibid.*, 650; B., 1942, II, 125.

[14] M. Dempsey, M. E. Robertson, A. E. Caunce, and W. R. Gaythwaite, *J. Soc. Leather Trades' Chem.*, 1942, **26**, 1; B., 1942, II, 173.

hides were stored at 36°.[15] G. L. Somer[16] has shown that the volatile nitrogen present in the hide before tanning can be used as an index of potential leather yields. A volatile nitrogen content greater than 3% indicates poor curing.

Liming.

The liming process continues to receive attention and a number of papers dealing with the effect of the addition of various compounds on the properties of lime liquors have appeared. E. R. Theis and R. O. Ricker[17] have measured the effect of the addition of a number of substances known to facilitate unhairing, viz., sodium sulphide, arsenic sulphide, sodium and calcium hydrosulphides, sodium sulphite, sodium cyanide, and arsenic oxide, on the pH and soluble calcium of lime liquors. The use of calcium hydrosulphide has received some publicity in the United States[18] and the unhairing action of lime liquors to which increasing amounts of this compound were added has been examined by E. R. Theis and W. A. Blum.[19] The rate of unhairing increased with the concentration until the resulting decrease in the solubility of the lime caused the pH to fall below 12·0. The use of methylamines in liming, also first initiated in the United States,[20] has recently been investigated in this country. In the liming of calf skins, dimethylamine was found to give more fine splitting and a more compact fibre weave than sodium sulphide.[21] In the liming of goatskins and the fellmongering of sheepskins, methylamines also caused more splitting up of the fibres and were found to produce a particularly fine grain.[22] The rate of unhairing decreased in the following order: sodium sulphide > monomethylamine > dimethylamine > trimethylamine > lime alone. The unhairing action of such a variety of substances, while at first sight confusing, is in agreement with the known chemistry of the disulphide linking, the scission of which is necessarily a preliminary to loosening of the hair. The disulphide linking is hydrolysed in alkaline solution, the extent of the hydrolysis increasing with pH: $R{\cdot}S{\cdot}S{\cdot}R + H_2O \rightarrow R{\cdot}S{\cdot}OH + R{\cdot}SH$. Further reactions, however, may take place leading to the formation of other cross-linkings between adjacent polypeptide chains and consequent increased stability of the keratin structure.

[15] L. M. Whitmore, G. V. Downing, and S. S. Sherrard, *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 41; B., 1942, II, 206.
[16] *Ibid.*, 34; B., 1942, II, 206.
[17] *Ibid.*, 1940, **35**, 663; B., 1942, II, 245. *Ibid.*, 1941, **36**, 201; B., 1941, II, 242.
[18] J. A. Wilson, *Shoe and Leather Rep.*, 1941, **223**, 12; B., 1942, II, 38.
[19] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 93; B., 1942, II, 245.
[20] G. D. McLaughlin and F. O'Flaherty, *ibid.*, 1927, **22**, 323; B., 1927, 972. G. D. McLaughlin, *ibid.*, 343; B., 1927, 972. G. D. McLaughlin, J. H. Highberger, and E. K. Moore, *ibid.*, 1928, **23**, 318; B., 1928, 681. E. K. Moore, J. H. Highberger, and F. O'Flaherty, *ibid.*, 1932, **27**, 2; B., 1932, 273.
[21] G. O. Conabere and E. W. Merry, *J. Soc. Leather Trades' Chem.*, 1942, **26**, 99; B., 1942, II, 269.
[22] J. H. Bowes and W. B. Pleass, *ibid.*, 140; B., 1942, II, 350.

The formation of cross-linkings by the following mechanisms has been suggested at various times:

(1) $R{\cdot}S{\cdot}OH + R'{\cdot}NH_2 \rightarrow R{\cdot}S{\cdot}NH{\cdot}R'$.[23]

(2) $R{\cdot}S{\cdot}OH \rightarrow H_2S + R{\cdot}CHO$[24] (at high pH values).
$R{\cdot}CHO + R'{\cdot}NH_2 \rightarrow R{\cdot}CH{:}N{\cdot}R'$.[25]

(3) $R{\cdot}CH_2{\cdot}S{\cdot}OH \rightarrow S + R{\cdot}CH_2{\cdot}OH$[26]
$R{\cdot}CH_2{\cdot}OH + R'{\cdot}SH \rightarrow R{\cdot}CH_2{\cdot}S{\cdot}CH_2{\cdot}R' + H_2O$.[27]

The possibility of ·C·S·C· linking was first suggested by J. B. Speakman and C. S. Whewell[28] and confirmatory evidence for its existence has now been obtained by M. J. Horn, D. B. Jones, and S. J. Ringel,[29] who report the isolation of lanthionine, $S[CH_2{\cdot}CH(NH_2){\cdot}CO_2H]_2$, by acid hydrolysis of wool which had been boiled for an hour with 2% sodium carbonate solution. A review of the present position with regard to the chemistry of the disulphide linking is given by J. L. Stoves,[29] together with further evidence for the ·C·S·C· linking.

The possibilities of the re-formation of cross-linkings as outlined above indicates that alkaline solutions alone will not readily unhair and offers an explanation of the difficulty encountered in unhairing hides which have been previously soaked in alkaline solutions.[30] Substances which can react with the sulphenic acid grouping and so prevent the re-formation of cross-linkings will facilitate unhairing, and the action of a number of such substances has been postulated as follows:

$R{\cdot}S{\cdot}OH + Na_2S \rightarrow R{\cdot}S^-Na^+$ + other sulphur-containing compounds, chiefly thiosulphate.[31]
$R{\cdot}S{\cdot}OH + NaHSO_3 \rightarrow R{\cdot}S{\cdot}SO_3^-Na^+$.[32]
$R{\cdot}S{\cdot}OH + NaCN \rightarrow R{\cdot}S^-Na^+ + R{\cdot}S{\cdot}CN$.[33]
$R{\cdot}S{\cdot}OH + NH_2Me \rightarrow R{\cdot}S{\cdot}NHMe$ [by analogy with (1)].
$R{\cdot}CHO + NH_2Me \rightarrow R{\cdot}CH{:}NMe$.[34]

The addition of a number of these substances has been shown to increase the pH of the lime solution[17,35] and hence will tend to increase the swelling of the collagen. When only small additions are made and the alkali-binding power of the collagen is taken into account, however, it is probable that the final pH of such liquors will not be very different from that of a lime liquor to which no addition has been made. The addition of the univalent sodium ion will, however, tend to increase the plumping.

[23] J. B. Speakman, *J. Soc. Dyers and Col.*, 1936, **52**, 335; B., 1936, 1033. *Nature*, 1936, **138**, 327; A., 1936, 1396.
[24] A. Schöberl, *Collegium*, 1936, 412; A., 1936, 1232.
[25] H. Phillips, *Nature*, 1936, **138**, 121; B., 1936, 489.
[26] A. Schöberl and P. Rambacher, *Annalen*, 1939, **538**, 84; A., 1939, II, 204.
[27] J. L. Stoves, *Trans. Faraday Soc.*, 1942, **38**, 254, 261; B., 1942, II, 366.
[28] *J. Soc. Dyers and Col.*, 1936, **52**, 380; B., 1936, 1198.
[29] *J. Biol. Chem.*, 1941, **138**, 141; A., 1941, II, 188.
[30] G. Vágó, *Collegium*, 1937, 512; B., 1938, 698.
[31] E. R. Theis, *J. Amer. Leather Chem. Assoc.*, 1941, **36**, 62; B., 1941, II, 167.
[32] F. F. Elsworth and H. Phillips, *Biochem. J.*, 1938, **32**, 837; A., 1938, III, 589.
[33] W. Windus and H. G. Turley, *ibid.*, 1941, **36**, 603; B., 1942, II, 86.
[34] W. B. Pleass, *Biochem. J.*, 1930, **24**, 1472; A., 1930, 1518. D. Jordan Lloyd, R. H. Marriott, and W. B. Pleass, *Trans. Faraday Soc.*, 1933, **29**, 554; A., 1933, 463.
[35] W. R. Atkin, L. Goldman, and F. C. Thompson, *J. Soc. Leather Trades' Chem.*, 1933, **17**, 568; B., 1934, 31.

This increase in plumping is not always desirable; there is some evidence that excessive plumping hinders the removal of the hair, since the hair roots are more firmly held,[36] and in the manufacture of leather in which a fine flat grain is required, it is generally considered that the plumping should be reduced to a minimum.

Of the substances enumerated above, five, calcium hydrosulphide, arsenic sulphide, and the methylamines, offer the possibility of accelerating the unhairing action of a lime liquor without at the same time increasing the plumping of the collagen and their use, therefore, would appear to offer certain advantages in the manufacture of some types of leather. Calcium hydrosulphide and arsenic sulphide may be considered to have the same effect, since the former is the active unhairing agent in arsenic limes.[37]

Grease in Skins and Leather.

R. M. Koppenhoefer[38] has reviewed previous work carried out by himself and co-workers on the distribution and composition of grease in hides and skins, its removal, and the application of greases in the fat-liquoring process.

The formation of calcium and chromium soaps during leather manufacture has been studied by R. F. Innes.[39] Calcium soaps formed from the free fatty acids in the skin during liming are decomposed by acids and vegetable tan liquors and hence can readily be removed. During chrome tanning they are converted into chromium soaps, the presence of which leads to uneven dyeing and greasiness in the finished leather. As chromium soaps are not readily decomposed or removed, the calcium soaps or free fatty acids giving rise to them should be removed before chrome tanning.

Vegetable Tanning.

Little further progress has been made in the elucidation of the structure of the tannins or the nature of the tannin–collagen reaction. The tanning action of a number of esters of gallic acid with polyhydric alcohols has been examined by A. Russell and W. G. Tebbens, jun.[40] The simple esters of gallic acid have no tanning action but polyesters show increased tanning action as the number of esterified hydroxyl groups in the alcohol increases. A. Küntzel[41] has surveyed his earlier work on the tanning action of organic tannins and from comparison of the effects of phenols and condensed phenols on collagen deduces that tanning involves reaction with the peptide groups of the collagen. R. O. Page[42] has made a study

[36] G. D. McLaughlin, J. H. Highberger, and E. K. Moore, *J. Amer. Leather Chem. Assoc.*, 1927, **22**, 345; B., 1927, 971. H. B. Merrill, *Ind. Eng. Chem.*, 1927, **19**, 386.
[37] E. Stiasny, *J. Soc. Leather Trades' Chem.*, 1919, **3**, 129. H. Herfeld, "Gerbereichemie und Lederfabrikation," Wien, Vol. II, p. 115.
[38] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 118; B., 1942, II, 270.
[39] *J. Soc. Leather Trades' Chem.*, 1941, **25**, 351; B., 1942, II, 86. *Ibid.*, 1942, **26**, 112; B., 1942, II, 303.
[40] *J. Amer. Chem. Soc.*, 1942, **64**, 2274; A., 1943, II, 61.
[41] *Kolloid-Z.*, 1942, **100**, 274; B., 1943, II, 162.
[42] *J. Soc. Leather Trades' Chem.*, 1942, **26**, 71; B., 1942, II, 125.

of the tannin–gelatin reaction. Experiments on the fractional precipitation of tannins with gelatin and salts indicate that tannin solutions contain molecules of varying size and that one molecule of tannin is precipitated by each basic group in gelatin, the larger molecules being precipitated first. It is shown that smaller molecules already combined with gelatin can be replaced by larger molecules. It is pointed out that the composition of the tannin–gelatin precipitate provides a possible method of following the changes in average size of the tannin molecules in the tanning processes.

The enzymic hydrolysis of valonia tannin with the production of ellagic acid and the deposition of "bloom" has been investigated by H. Anderson and S. O. Sourlangas[43]; the enzyme causing the hydrolysis is specific to valonia and divi-divi. Factors influencing the fixation of quinone by collagen have been studied by H. C. Stecker and J. H. Highberger[44]; the amount fixed is dependent on the pH and the extent of oxidation and polymerisation of the quinone.

No doubt stimulated by the war and the possible shortage of tanning materials, a number of papers have appeared dealing with the properties of the less common tannins.[45]

Mineral Tanning.

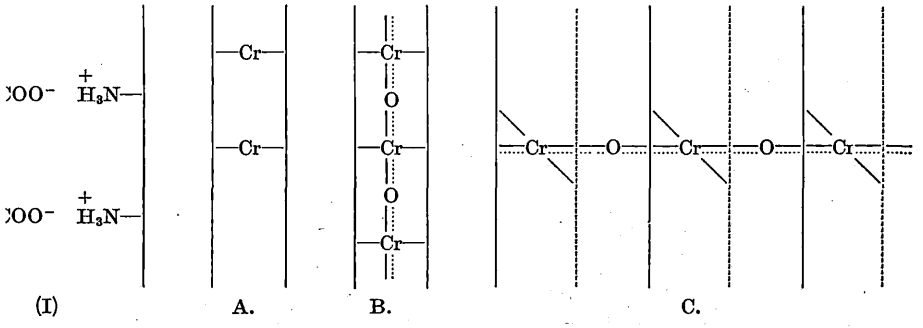
E. Elöd[46] and A. Küntzel[47] have published papers summarising their work with various collaborators during the past few years. Both have used changes in absorption spectra during the reaction between metallic salts and gelatin and glycine to throw light on the mechanism of the tanning process. This is not a new technique as suggested in a previous Report[48] but has been used for some years.[49,50] A. Küntzel and C. Riess[50] consider that the reaction between chromium and gelatin is the same in principle as that between chromium and glycine. E. Elöd and T. Schachowskoy,[46,51] however, criticise this statement on the grounds that their observations with gelatin, glycine, and other substances containing amino- and carboxyl groups do not justify this assumption. They consider that conclusions regarding the reactions taking place with collagen cannot be drawn from the changes in light absorption occurring in the reaction of chromium with partly hydrolysed gelatin. A. Küntzel[47,52] has replied to certain of these criticisms, one of his contentions

[43] *J. Soc. Leather Trades' Chem.*, 1942., **26**, 49; B., 1942, II, 245.
[44] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 226; B., 1942, II, 350.
[45] J. S. Rogers and C. O. Beebe, *ibid.*, 1941, **36**, 525; B., 1942, II, 39. R. W. Frey and I. D. Clarke, *ibid.*, 576; B., 1942, II, 86. L. Baens, *Philippine J. Sci.*, 1941, **75**, 363; B., 1942, II, 173. L. G. Alego, L. Baens, and V. G. Lava, *ibid.*, **76**, 1; B., 1942, II, 125.
[46] E. Elöd and T. Schachowskoy, *Kolloid Beih.*, 1939, **51**, 1; B., 1942, II, 390.
[47] A. Küntzel, *Kolloid-Z.*, 1940, **91**, 168.
[48] M. P. Balfe, *Ann. Repts.*, 1941, **26**, 404.
[49] E. Elöd and T. Schachowskoy, *Trans. Faraday Soc.*, 1935, **31**, 216; A., 1935, 301.
[50] A. Küntzel and C. Riess, *Collegium*, 1936, 138; B., 1936, 754.
[51] E. Elöd and T. Schachowskoy, *Kolloid-Z.*, 1941, **94**, 328, 333; B., 1942, II, 173.
[52] *Collegium*, 1940, 106; B., 1940, 555. *Kolloid-Z.*, 1941, **97**, 99; B., 1942, II, 350.

being that any differences in the light absorption curves for the two systems chromium–glycine and chromium–gelatin can be accounted for by the spatial arrangement of the amino- and carboxyl groups in the two systems.

The evidence available on the theory of mineral tanning as a result of the investigations of these and other workers favours a chemical rather than a physical explanation and on this basis a reasonable hypothesis can be built up involving the formation of cross-linkings between adjacent polypetptide chains by reaction of the metallic complexes with active centres in the collagen molecule. In spite of the arguments put forward by E. Elöd,[46,51] the balance of evidence favours the suggestion that combination with the carboxyl groups of the collagen followed by the formation of a further co-ordinate linking with the free amino-groups are the main reactions involved in tanning. The possibility that other groups are involved, as suggested by a study of the reaction of polyvinyl alcohols with a variety of metallic salts,[46] cannot, however, be excluded.

The mechanism put forward by A. Küntzel and C. Riess[50] to account for the thermal stability of chrome-tanned leather is attractive as a working hypothesis. The polypeptide chains of the collagen, with side-chains terminating in free carboxyl and amino-groups, are represented diagrammatically as in (I) and combination with chromium or other suitable metallic complexes is postulated as shown in A, B, and C. It



(I) A. B. C.

is suggested that the formation of polynuclear chromium complexes is essential for a tanning action to take place, since only such salts are sufficiently extended in space to be able to react with several pairs of carboxyl and amino-groups in different polypeptide chains, so forming a lattice-like structure. A. Küntzel and T. Droscher[53] suggest that for this reason copper salts, which do not readily form molecules containing several metallic atoms, have no tanning action, although it is known that they form stable complexes with glycine. It is interesting to consider the possibilities of formulæ of this type in relation to the stereochemical configuration of the collagen molecules. It can be calculated that the distances involved in the formation of bridges as postulated above, with

[53] *Collegium*, 1940, 122; B., 1940, 535.

the exception of (B), do not require the existence of complexes containing more than 3 or 4 chromium atoms.

A number of papers have appeared recently dealing with the effect of added anions on the chrome tanning process.[54–56] The results obtained by different workers are often contradictory, probably owing to the fact that in many cases other conditions have not been adequately controlled. For instance, in some cases the effect of the added salt on the pH of the chrome liquor has not been distinguished from the specific effect of the salt itself.[54] Since the majority of the salts studied are those of organic acids the effect on the pH is likely to be appreciable. The amount of the salt added also influences the results.[54,55] If the anion of the salt is capable of forming a stable complex with chromium, the addition of amounts equivalent to the chromium content of the solution may preclude any reaction of the chromium with the collagen. It is probable that when pH and other conditions are satisfactorily standardised many of the seeming contradictory effects of the addition of salts will disappear.

The effect of salts on the chromium uptake may in part be ascribed to the formation of complexes between the chromium and the anion in question. On the assumption that the formation of similar complexes between the metallic cations and the free carboxyl groups takes place during tanning it may be expected that the effect of different anions on the chromium uptake will be dependent on the relative stability of the complexes formed between the anions and chromium compared with the stability of the chromium–collagen complex.

The addition of salts to the chrome liquor affects the thermal stability of the resulting leather as well as its chromium content.[54,55,57,58] An explanation of this effect is given by A. Küntzel and C. Riess.[58] They consider that under the conditions of chrome tanning, where the majority of the amino-groups are positively charged, the formation of stable bonds between these groups and the chromium atoms in the complex is unlikely and, therefore, the formation of stable cross-linkings is dependent on the presence of a suitable anion in the complex capable of forming a co-ordinate linking with the charged amino-groups.

Tanning with other mineral salts has also received attention. E. Elöd and T. Schachowskoy[46] relate the pH of optimum tanning action of a number of metallic salts to the pH at which the hydroxide of the metal is precipitated. E. O. Wilson and S. C. Yu[59] have studied the tanning action

[54] H. C. Holland, *J. Soc. Leather Trades' Chem.*, 1940, **24**, 152, 199, 221; B., 1940 554, 631, 688.
[55] E. R. Theis and J. Ganz, *J. Amer. Leather Chem. Assoc.*, 1940, **35**, 504; B., 1940, 815. E. R. Theis and W. K. Meerbott, *ibid.*, 1942, **37**, 293; B., 1942, II, 206. E. R. Theis, *ibid.*, 1941, **36**, 449; B., 1942, II, 447.
[56] G. D. McLaughlin and R. S. Adams, *ibid.*, 1942, **37**, 76; B., 1942, II, 246.
[57] K. H. Gustavson, *Stiasnyfestschrift*, 1937, 99; B., 1938, 304.
[58] *Collegium*, 1936, 635; B., 1937, 68.
[59] *J. Physical Chem.*, 1941, **45**, 167; B., 1941, II, 168.
[60] *Kolloid-Z.*, 1941, **97**, 336; B., 1942, II, 350.
[61] *J. Soc. Leather Trades' Chem.*, 1942, **26**, 44; B. ,1942, II, 206.

of aluminium sulphate and consider that the fixation of aluminium by collagen may be represented by exponential curves of the Freundlich adsorption isotherm type. T. Schachowskoy and H. G. Fröhlich[60] distinguish between two types of zirconium tannage, a "salt" tannage which takes place with zirconium sulphate but not with the nitrate or chloride and having a maximum between pH 2 and 3, and a hydroxide tannage which is independent of the anion and reaches a maximum between pH 5 and 6.

The fate of the aluminium used in pickling prior to chrome tanning has been investigated by H. E. Herman, J. R. Blockey, C. H. Spiers, and A. J. Stoneman.[61] It was found that chrome tanning removes more aluminium from the skin than washing with water and that the chrome-tanned leather retained only 15% of the aluminium used for pickling.

Properties of Leather.

The determination of the density of leather and its relation to other properties such as water absorption and thermal conductivity are the chief topics which have aroused interest. W. Gallay and J. S. Tapp[62] have devised a new method based on Boyle's law for determining the real density of leather. The leather is introduced into a vessel connected to a mercury manometer and the difference in pressure required to bring the mercury to the same level in the connecting tube before and after the introduction of the leather is used to calculate the real volume of the leather. This calculation involves the assumption that the pressure of the air in the pores of the leather can come into equilibrium with that of air in the surrounding vessel. It is claimed that this method is simpler and gives greater accuracy than the methods involving the displacement of the air in the leather with paraffin.[63] N. W. McLachlan,[64] following on an earlier paper[65] on the thermal conductivity of shoe materials, has related this property to the apparent density of the leather, and values for many types of leathers are given. H. C. Holland[66] has described a method by which the water absorbed by leather can be divided into two fractions, the combined water and the water present in the free spaces. The "free space water" is assumed to be equal to the air spaces in leather as measured by displacement with kerosene. There is some error in this assumption, since the water will cause the fibres to swell, thereby reducing the space available, and at the same time remove water soluble material and so increase this space. Holland considers that the total error introduced is small since the two errors are compensating. The effect of various tanning processes on the water absorbed in these two ways has been determined.

[62] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 140; B., 1942, II, 270.

[63] R. E. Porter, *ibid.*, 1929, **24**, 36. I. D. Clarke, *J. Ind. Eng. Chem.*, 1931, **23**, 62; B., 1931, 409.

[64] *J. Soc. Leather Trades' Chem.*, 1942, **26**, 135; B., 1942, II, 351.

[65] N. W. McLachlan, T. R. G. Lewis, and G. Goodfellow, *ibid.*, 1941, **25**, 76; B., 1941, II, 207.

[66] *Ibid.*, 1942, **26**, 124; B., 1942, II, 303.

The effect of different tanning materials on the "free water" (the water lost by a sample of leather saturated with water on drying out to equilibrium at 75% R.H. expressed as a percentage of the original dry weight) and the moisture content of sole leather has been determined by G. H. W. Humphreys.[67] The "free water" values are not only dependent on the amount of water-soluble material and the amount of open space in the leather, but are also affected by the nature of the tanning material, myrobalan in particular tending to give low values. Myrobalan-tanned leather was also markedly less hygroscopic than the rest of the leathers, although this tanning material itself was the most hygroscopic of those examined.

G. O. Conabere[68] has continued her work on the measurement of the "feel" of leather and has extended it to the numerical evaluation of the properties of firmness and fullness. Values obtained for a number of chrome calf upper leathers are given. The relationship between the property of gloving leathers usually referred to as "run" and other physical properties has been examined.[69] R. B. Hobbs and P. E. Tobias[70] have measured some physical and chemical properties of belting leather and L. Boor and J. G. Niedercorn[71] have used the Olsen bursting tester for the evaluation of the strength of leather.

In an investigation designed to developed a suitable ageing test for leather J. R. Kanagy[72] has measured the evolution of carbon dioxide and water from vegetable-tanned leathers at elevated temperatures; the amounts of carbon dioxide evolved are related to the comparative stability of the leathers.

[67] *J. Soc. Leather Trades' Chem.*, 1942, **26**, 59; B., 1942, II, 246.
[68] *Ibid.*, 1941, **25**, 319; B., 1942, II, 126.
[69] W. B. Pleass, *ibid.*, 1942, **26**, 152; B., 1942, II, 390. J. H. Bowes, *ibid.*, 181, 189; B., 1942, II, 448.
[70] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 131; B., 1942, II, 270.
[71] *Ibid.*, 178; B., 1942, II, 303.
[72] *Ibid.*, 1941, **36**, 609; B., 1942, II, 39.

LEATHER

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LEATHER.

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During the past year there has been some criticism of the quality of the sole leather being used on civilian footwear. Authoritative statements on the subject have been made by B. H. Harvey[1] and D. Jordan Lloyd,[2] who point out that the bulk of the best sole leather produced in this country is needed for the armed forces, and that there is no evidence that the standard of English sole leather has deteriorated. In spite of war-time shortages of certain materials, the increased knowledge of the tanning process and its control, which has been obtained since the last war, has made it possible to maintain, and even in some cases to surpass, the pre-war standards.

There are fewer papers than usual this year dealing directly with the production of leather and its properties. In the past few years, however, a number of papers have appeared dealing with the amino-acid composition of the proteins and the opportunity has, therefore, been taken to review the advances in this branch of protein chemistry in so far as they have a bearing on the structure and composition of collagen and the changes which are likely to occur during the processes involved in leather production.

Collagen.

It is becoming increasingly evident that there is need for more accurate determinations of the amino-acid composition of proteins,[3,4] in view of their bearing on the theories of protein structure which have been based on *X*-ray crystallography or which deal with the arrangement of the amino-acids in individual proteins.[5] The Bergmann–Niemann hypthesis[5] concerning the orderly arrangement of the amino-acids in the protein molecule has recently received some criticism. A. C. Chibnall[4] discusses the structure of a number of proteins in the light of the amino-acid analyses at present available and considers that it would be wise to regard the Bergmann–Niemann hypothesis as still tentative. It has also been suggested that, considering the errors at present involved in the determination of the amino-acids, there is a high probability that a purely random distribution of amino-acids would give values in apparent accordance with this hypothesis.[6] A. G. Ogston[7] has considered the hypothesis

[1] *Leather Tr. Rev.*, 1943, **76**, 913.
[2] *Ibid.*, 1015. *Leather World*, 1943, **35**, 566.
[3] W. T. Astbury, *J.C.S.*, 1942, 37; A., 1942, I, 231.
[4] A. C. Chibnall, *Proc. Roy. Soc.*, 1942, **B**, **121**, 136; A., 1943, II, 75.
[5] M. Bergmann, *J. Biol. Chem.*, 1935, **110**, 471; A., 1935, 1140 M. Bergmann and C. Niemann, *ibid.*, 1936, **115**, 77; 1937, **118**, 301; 1938, **122**, 577; A., 1936, 1283; 1937, III, 168; 1938, III, 210; *Science*, 1937, **86**, 187; A., 1937, III, 41.
[6] A. Neuberger, *Proc. Roy. Soc.*, 1939, **B**, **127**, 25; A., 1939, III, 785. N. W. Pirie, *Ann. Repts. Chem. Soc.*, 1939, **36**, 352.
[7] *Trans. Faraday Soc.*, 1943, **39**, 151; A., 1943, II, 247.

from a mathematical viewpoint and concludes that there is no suggestion that the numbers 2 and 3 hold any unique position as prime factors in the structure of a regular array, and that if the Bergmann–Niemann hypothesis should prove to be correct, deeper insight into the structure and mode of formation of proteins will be needed to explain it.

H. Neurath[8] points out that the arrangement of the amino-acids in proteins is also likely to be dependent on stereochemical considerations and suggests that it is significant that gelatin, which contains a high proportion of proline and hydroxyproline (both having bulky side-chains), contains a correspondingly high proportion of glycine.

Direct evidence for the arrangement of the amino-acid residues in a peptide chain can only be obtained by the isolation and identification of partial hydrolysis products containing the necessary minimum number of residues.[9] A. H. Gordon, A. J. P. Martin, and R. L. M. Synge have carried out researches on these lines using chromatographic methods.[9,10,11] Evidence arising from their experiments with gelatin[11] suggest that the residues of the basic amino-acids are directly linked to the higher monoamino-acids and that the monoamino-acids with long side-chains are not linked to one another in the polypeptide chain. A study of the course of the hydrolysis suggested that certain peptide links were more resistant to acid hydrolysis than others.

The values usually quoted for the amino-acid constituents of collagen and gelatin are mainly those obtained by H. D. Dakin[12] some twenty years ago and apart from some analyses by F. Schneider[13] and the determination of a few of the individual amino-acids by other workers there are no more recent figures available.

The older methods of analysis which were largely used in these determinations involve a number of separations followed by gravimetric determination of the amino-acid in question. Such precipitation methods are subject to errors due to solubility factors which may vary according to the amino-acid mixture present in the hydrolysate. Even in the case of the basic and acidic amino-acids, which are the most readily isolated, it is realised that the results are not highly accurate. A. C. Chibnall, M. W. Rees, and E. F. Williams[14] have carried out very careful analyses of the acidic and basic amino-acids of edestin, egg-albumin, and β-lactoglobulin in which each step in the analysis was carefully checked and the errors involved were known within small limits. The values obtained for the dicarboxylic acid content of these and other proteins are considerably higher (in some cases 50% higher) than values previously

[8] *J. Amer. Chem. Soc.*, 1943, **65**, 2039; A., 1944, II, 88.
[9] *Biochem. J.*, 1941, **35**, 1369; A., 1942, II, 158.
[10] A. J. P. Martin and R. L. M. Synge, *ibid.*, 1358; A., 1942, II, 160. A. H. Gordon, A. J. P. Martin, and R. L. M. Synge, *ibid.*, 1943, **37**, 79, 86, 313; A., 1943, II, 179.
[11] A. H. Gordon, A. J. P. Martin, and R. L. M. Synge, *ibid.*, 92; A., 1943, II, 179.
[12] *J. Biol. Chem.*, 1920, **44**, 499.
[13] *Collegium*, 1940, 97; B., 1940, 554.
[14] *Biochem. J.*, 1943, **37**, 372; A., 1943, II, 346. K. Bailey, A. C. Chibnall, M. W. Rees, and E. F. Williams, *ibid.*, 361; A., 1943, II, 404.

obtained by H. D. Dakin[15] and others. By analogy, therefore, it seems probable that the values obtained by Dakin[12] for gelatin are correspondingly lower than the true values. An analysis of collagen using these more refined techniques would, therefore, be welcome.

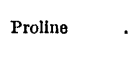
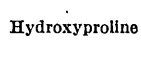
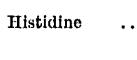
Two new techniques, which largely avoid the errors inherent in the old gravimetric methods have recently been developed, the solubility product method of M. Bergmann[16,17] and the chromatographic methods of Gordon, Martin, and Synge.[10,11] The glycine and proline content of collagen and gelatin have been determined by the solubility product method[16] and valine has been isolated from gelatin for the first time using the chromatographic technique.[11]

With regard to the determination of individual amino-acids, L. A. Shinn and B. H. Nicolet have recently developed a method for the determination of the hydroxyamino-acids, threonine[18] and serine,[19] in proteins, based on the determination of the amounts of acetaldehyde and formaldehyde formed by the addition of periodic acid. It is suggested that the reaction takes place as follows: $R{\cdot}CH(OH){\cdot}CH(NH_2){\cdot}CO_2H + O \longrightarrow R{\cdot}CHO + NH_3 + CHO{\cdot}CO_2H (+ O) \longrightarrow CO_2 + HCO_2H$. They report the presence of much greater amounts of these acids than have hitherto been isolated; in gelatin they found 1·4% of threonine and 3·2—3·4% of serine. D. D. Van Slyke, A. Hiller, and D. A. MacFadyen[20] have adapted the same technique to the determination of hydroxylysine after previous separation from the other known hydroxy-acids; in this case the ammonia evolved on the addition of periodic acid was measured. Gelatin and collagen were the only proteins which contained an appreciable amount of hydroxylysine (0·7—0·9%).

In the table on page 324 the amounts of the different amino-acids which have been isolated from gelatin and collagen at various times are listed. With many of the amino-acids, values for gelatin only have been obtained, and in view of the information now available regarding the lability of certain groups in the protein molecule on treatment with acid or alkali, it is open to question whether the values are in all cases applicable to collagen unless there is adequate knowledge of the previous treatment of the gelatin. In the fourth column of the table are given "selected" values for the amounts of the different amino-acids present in collagen. These have been selected from the various analytical results, either by reason of the technique used, or because they represent the average value of a number of determinations by different workers.

[15] *Biochem. J.*, 1918, **12**, 290.
[16] M. Bergmann and W. H. Stein, *J. Biol. Chem.*, 1939, **128**, 217; A., 1939, II, 231.
[17] W. H. Stein, M. Bergmann, and C. Niemann, *J. Amer. Chem. Soc.*, 1938, **60**, 1703; A., 1938, II, 386. H. R. Ing and M. Bergmann, *J. Biol. Chem.*, 1939, **129**, 603; A., 1939, II, 463. W. H. Stein and M. Bergmann, *ibid.*, 1940, **134**, 627; A., 1940, II, 320. M. Bergmann, S. Moore, and W. H. Stein, *Chem. Rev.*, 1942, **30**, 423. S. Moore and W. H. Stein, *J. Biol. Chem.*, 1943, **150**, 113; A., 1943, II, 403.
[18] *Ibid.*, 1941, **138**, 91; A., 1941, II, 208.
[19] *Ibid.*, 1941, **139**, 687; A., 1941, II, 308.
[20] *Ibid.*, **141**, 681; A., 1942, II, 184.

Composition of Collagen and Gelatin.

Amino-acid.	Side-chain*	Mol. wt.	g. per 100 g. protein. Gelatin.	Collagen.	Selected value for collagen.
Neutral amino-acids.					
Glycine	H	75	25·5[13] 26·5[16] 25·6[21] 25·5[22]	24·7[13] 27·2[13] 26·2—26·7[16]	26·5
Alanine	CH_3·	89	8·7[13]		8·7
Phenylalanine ..	C_6H_5·CH_2·	165	1·4[13] 1·2[23]		1·3
Leucine	$(CH_3)_2CH$·CH_2·	131	7·1[13]		7·1
Valine	$(CH_3)_2CH$·	117	1·7[11]		1·7
Methionine	CH_3·S·$[CH_2]_2$·	149	0·07[24]		0·07
Proline		115	19·7[13] 17·6[16] 19·7[23]	17·5[16] 18·9[13]	17·5
Hydroxyproline ..		131	14·1[13] 14·4[25]	10·8[13]	14·2
Serine	OH·CH_2·	105	0·4[13] 3·43†[13]		3·4
Threonine	CH_3·CH(OH)·	119	1·4[16]		1·4
Tyrosine	*p*-OH·C_6H_4·CH_2·	181	0·3[25]	0·8[25] 1·0[13]	1·0
Basic amino-acids.					
Lysine	NH_2·$[CH_2]_4$·	146	5·9[13]	4·7[13] 5·0[13] 3·8—4·1[26]	4·8
Hydroxylysine ..	NH_2·$[CH_2]_3$·CH(OH)·	162	0·73—0·90[20]	0·88[20]	0·9
Arginine	NH: C(NH_2)·NH·$[CH_2]_3$·	174	8·2[13] 8·3[13] 8·7[27]	8·4[13] 8·1[13] 7·7[26]	8·1
Histidine		155	0·9[13]	0·5—0·6[13] 0·4[26]	0·5
Acidic amino-acids.					
Aspartic acid‡ ..	HO_2C·CH_2·	133	3·4[13]	3·5[13]	3·5
Glutamic acid‡ ..	HO_2C·$[CH_2]_2$·	147	5·8[13]	5·7[13]	5·7
Amide nitrogen (as NH_3)			0·4[13]		
				Total	107·3

* R in R·CH(NH_2)·CO_2H except in case of proline and hydroxyproline where the formula is given.
† This figure also includes hydroxylysine.
‡ May be present as amides.

The figures given are the percentages of the amino-acids isolated from the protein, and in order to allow for the water added during hydrolysis the sum of these percentages should add up to about 118. The isolation of all the amino-acids in collagen and gelatin is, therefore, not complete,

[21] B. W. Town, *Biochem. J.*, 1936, **30**, 1833; A., 1936, 1528.
[22] M. Bergmann, *J. Biol. Chem.*, 1935, **110**, 471; A., 1935, 1140.
[23] R. Kapeller-Adler, *Biochem. Z.*, 1932, **252**, 185; A., 1932, 1150.
[24] H. D. Baernstein, *J. Biol. Chem.*, 1932, **97**, 663; A., 1932, 1149.
[25] O. Gerngross, K. Voss, and H. Herfeld, *Collegium*, 257, 1933; A., 1933, 407.
[26] J. H. Highberger, *J. Amer. Leather Chem. Assoc.*, 1938, **33**, 9; B., 1938, 414.
[27] H. B. Vickery, *J. Biol. Chem.*, 1940, **132**, 325; A., 1940, II, 200.

there still remaining about 10% to be accounted for. In view of the work of A. C. Chibnall and co-workers[14] on other proteins, it seems probable that this deficiency is, at least in part, due to the incomplete isolation of the dicarboxylic acids.

The leather chemist is concerned not only with the composition of collagen, but also with the changes it may undergo in the liming, bating, and pickling processes which precede tanning.

Liming.—It has been suggested that the following changes may take place during liming: (*a*) Hydrolysis of the amide groups of asparagine and glutamine residues to give free carboxyl groups.[28] (*b*) Modification of the guanidine group of arginine with the production of urea.[28] (*c*) Loss of the amino-acid lysine.[29]. (*d*) Hydrolysis of the peptide groups with the liberation of carboxyl and amino-groups.

The isolectric point is shifted towards the acid range by liming or other alkaline treatment[30] and this shift may be largely attributed to reaction(*a*). The work of R. C. Warner[31] on the kinetics of the hydrolysis of arginine by alkali suggests that ornithine may be produced from this amino-acid during liming:

$$>CH\cdot[CH_2]_3\cdot NH\cdot C(:NH)\cdot NH_2 \begin{cases} \xrightarrow{1} CO(NH_2)_2 + >CH\cdot[CH_2]_3\cdot NH_2 \\ \xrightarrow{2} NH_3 + >CH\cdot[CH_2]_3\cdot NH\cdot CO\cdot NH_2 \end{cases}$$

The second reaction also takes place but to a smaller extent.

R. C. Warner and R. K. Cannan[32] have also found that the ammonia produced by the action of alkali on proteins exceeds that which can be attributed to the amide and arginine groups and suggest that this is connected with labile groups, possible in serine and threonine. The work of B. H. Nicolet, L. A. Shinn, and L. J. Saidle[33] also indicates that serine and threonine are affected by alkali treatment. On subsequent acid hydrolysis the amounts of these amino-acids are reduced and there is a corresponding increase in the amide nitrogen.

E. R. Theis and T. F. Jacoby[34] have investigated the effect of liming on the acid- and base-combining capacity of collagen. They confirm that the isoelectric point is shifted to lower pH-values and the shape of their curves is in agreement with the suggestion that hydrolysis of the amide groups takes place during liming. The acid- and base-combining capacity increased as liming proceeded, suggesting that additional acidic and basic groups were liberated.

[28] J. H. Highberger and H. C. Stecker, *J. Amer. Leather Chem. Assoc.*, 1941, **36**, 368; B., 1941, II, 406.
[29] E. R. Theis and T. F. Jacoby, *ibid.*, 375; B., 1941, II, 405.
[30] J. R. Beck and A. M. Sookne, *J. Res. Nat. Bur. Stand.*, 1939, **23**, 271; A., 1939, I, 544. J. H. Highberger, *J. Amer. Chem. Soc.*, 1939, **61**, 2302; A., 1939, I, 564. E. C. E. Hunter and A. J. Turner, *Trams. Faraday Soc.*, 1940, 835; A., 1940, I, 358.
[31] *J. Biol. Chem.*, 1942, **142**, 705; A., 1942, I, 243.
[32] *Ibid.*, 725; A., 1942, II, 241.
[33] *Ibid.*, 609; A., 1942, II, 211.
[34] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 611; B., 1943, II, 252.

Bating and Pickling.—Little work has been done on the chemical aspects of bating, but evidence obtained regarding the action of trypsin on various proteins indicate that the liberation of arginine from the collagen is likely to take place.[35] The acid used in conjunction with sodium chloride in the pickling process is not likely to cause any appreciable change in the composition of the collagen unless excessive amounts of acid are used, the skins stored for long periods in the pickled condition, or the temperature raised above 25° C.[36] Under these conditions there is an increase in the water-soluble nitrogen, suggesting that some degradation has taken place.

E. R. Theis and T. F. Jacoby[34,37,38] have continued their investigations on the effect of various factors on the acid- and base-combining capacity of collagen. They have examined the effects of liming[34] (see above), the addition of potassium and calcium chloride,[37] and of previous heating on the shape of the titration curves.[38] They find that in the presence of calcium chloride, the curves are shifted to lower pH values in the range 4·5—7·5. They also find that appreciably more calcium than potassium ions are bound by the protein. Previous heating in water to 60° C. had no affect on the maximum acid- and base-combining capacity but in the pH range 4·5—10·0 the curves were shifted to higher pH values. They suggest that the change in shape of the curves in this case may be due to the conversion of certain electrovalent salt linkings into co-ordinate linkings. These authors[39] also discuss the significance of the shrinkage temperature of collagen. They have studied the effects of acids and salts on the stability of collagen as measured by the shrinkage temperature and consider that resistance to shrinkage is due to salt linkings and hydrogen bonds between adjacent polypeptide chains.

The structures for collagen and other fibrous proteins put forward by W. T. Astbury[40] have been criticised by M. L. Huggins,[41] who suggests rather different structures which he claims fit the *X*-ray and analytical data better.

F. O. Schmitt, C. E. Hall, and M. A. Jakus[42] have extended their work with the electron microscope and have examined collagen obtained from a variety of sources at magnifications up to 28,000. In all cases the fibres showed characteristic cross striations of alternate opaque and transparent bands about 440 and 200 A. respectively in width. The distance between these striations varied from about 400 to 900 A. but the most frequently occurring spacings lay between 620 and 660 A., values agreeing very

[35] J. A. Dauphinee and A. Hunter, *Biochem. J.*, 1930, **24**, 1128; A., 1930, 1317. S. Sakaguchi, *J. Biochem, Japan*, 1925, **5**, 159.
[36] J. H. Bowes, *Leather World*, 1942, **34**, 398, 484; B., 1942, II, 303, 389.
[37] *J. Biol. Chem.*, 1943, **148**, 603; A., 1943, I, 226.
[38] *Ibid.*, 105; A., 1943, III, 517.
[39] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 433; B., 1943, II, 32.
[40] W. T. Astbury, *J. Soc. Leather Trades' Chem.*, 1940, **24**, 69; B., 1940, 383. W. T. Astbury and F. O. Bell, *Nature*, 1940, **145**, 421; 1941, **147**, 696; A., 1940, I, 199; 1941, II, 34.
[41] *Chem. Rev.*, 1943, **32**, 195.
[42] *J. Cell. Comp. Physiol.*, 1942, **20**, 11; A., 1943, III, 80.

closely with that for the long-chain X-ray spacing found by R. S. Bear in the same laboratories.[43] The fibrils could be very greatly extended under tension, the spacings increasing to values greater than 6000 A., this extension mainly taking place in the transparent bands. The author suggest that this elongation is due to unfolding of the linear elements or protofibrils forming the collagen fibril and that this coiling must be very much more extensive than that pictured by W. T. Astbury[40] as characteristic of α-keratin.

Processes Preceding Tanning.

W. T. Roddy[44] has made a histological study of the distribution of the coagulable proteins in various types of skins and the changes produced during soaking, liming, and other pretanning processes. He also considers methods of removing the coagulable protein and its effect on the physical properties of the finished leather.

The disinfection of hides suspected of contamination with the virus of foot and mouth disease continues to receive consideration. Sodium bifluoride and sodium silicofluoride in concentrations of 1 in 5000 to 1 in 10,000 have been found to kill the virus of vesicular stomatitis in twenty-four hours.[45] This virus, although closely related to that of foot and mouth disease, is non-pathogenic and there may, therefore, be some doubt whether the results obtained with it can be applied to the foot and mouth problem.

The chemistry of the unhairing process was reviewed in last year's Report.[46] During the past year M. J. Horn, D. B. Jones, and S. J. Ringel[47] have produced further evidence for the isolation of lanthionine from the acid hydrolysates of various alkali-treated proteins. L. R. Mizell and M. Harris[48] have isolated lanthionine equivalent to 25—28% of the cystine lost by wool during alkaline treatment. These authors discuss the various theories put forward regarding the reaction of wool with alkali. They consider that these are not in agreement with certain of the experimental facts such as the failure to confirm the presence of thiol and aldehyde groups in alkali-treated wool, and they are of the opinion that the formation of lanthionine is one of the principal reactions involved.

E. R. Theis and W. A. Blum[49] have studied the effect of pH, alkali, and pretreatment with sulphides or cyanides on the destruction and loss sulphur from hair during liming.

[43] *J. Amer. Chem. Soc.*, 1942, **64**, 727; A., 1942, I, 231.
[44] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 410, 416; B., 1943, II, 32.
[44] W. T. Roddy and R. P. Hermosa, *ibid.*, 1943, **38**, 98; B., 1943, II, 266.
[45] C. A. Manthei and A. Eichhorn, *J. Agric. Res.*, 1941, **63**, 41; B., 1942, II, 415.
[46] J. H. Bowes, *Ann. Repts.*, 1942, **27**, 357.
[47] *J. Biol. Chem.*, 1942, **144**, 87, 93; A., 1942, II, 338.
[48] *J. Res. Nat. Bur. Stand.*, 1943, **30**, 47; *Amer. Dyestuff Rep.*, 1943, **32**, 145; B., 1943, II, 249.
[49] *J. Amer. Leather Chem. Assoc.*, 1943, **38**, 68; B., 1943, II, 266.

TANNING.

Vegetable Tanning.

Interest has been taken in the possibilities of extending the sources of tanning materials available in the United States. A survey has been made of the natural tanning materials of the South-Eastern United States[50] in which the distribution and tannin content of a wide variety of trees and shrubs have been examined. The sources of vegetable tannins in Florida have also been considered.[51] I. B. Clarke, E. T. Sterner, and R. W. Frey[52] find that the tannin content of Chinese chestnut grown in Maryland increases with age and its distribution becomes more uniform.

The threatened shortage of hydrolysable (pyrogallol) tannins is probably the cause of recent interest in the properties of this type of tannin. W. R. Atkin and S. D. Sourlangas[53] have determined the titration curves of a number of the hydrolysable tannins before and after extraction with ether. On the supposition that extraction with ether only removes the non-tannin acids, they conclude that the tannin molecules of chestnut, valonia, myrobalan, divi-divi, and algarobilla contain a free carboxyl group whereas those of gallotannic acid and sumach do not. They attribute the acidity of gallotannic acid solutions to the presence of gallic acid as an impurity.

The acidic properties of the hydrolysable tannins can in part be reproduced by the addition of acids to the condensed tannins and the production of a substitute for chestnut wood extract, by the addition of citric or tartaric acids to mimosa extract, has been described.[54] D. Jordan Lloyd[55] draws attention to the possible effect of the deposition in the leather of ellagic acid derived from the pyrogallol tannins during layering and hot-pitting. Factors governing the deposition of ellagic acid from valonia have been examined by H. Anderson and S. D. Sourlangas.[56]

E. D. Compton[57] has commenced a study of the particle size of tannin solutions by ultra-filtration methods. In solutions of ordinary quebracho he finds no change in particle size either with concentration of tannin or on the addition of synthetic tannins.

The hypotheses which have been put forward with regard to the mechanism of the fixation of vegetable tannins by collagen were discussed in these Reports two years ago.[58] The work published since that date tends to favour an adsorptive mechanism. A. Cheshiré[59] has studied the equilibrium between vegetable-tanned leathers and water and the

[50] A. Russell, *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 340; B., 1942, II, 416. *Ibid.*, 1943, **38**, 30, 144, 235, 355; B., 1943, II, 232, 267, 334.
[51] W. D. May and E. E. Frahm, *ibid.*, 210; B., 1943, II, 330.
[52] *Ibid.*, 1942, **37**, 591; B., 1943, II, 233.
[53] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 183; B., 1943, II, 362.
[54] R. Hodgson and Sons, Ltd., and A. Cheshire, B.P. 548,594; B., 1943, II, 34.
[55] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 20; B., 1943, II, 163.
[56] *Ibid.*, 1942, **26**, 49; B., 1942, II, 245.
[57] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 486, 598; B., 1943, II, 67, 233.
[58] M. P. Balfe, *Ann. Repts.*, 1941, **26**, 356.
[59] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 123, 145; B., 1943, II, 362.

desorption of tannin from leathers in solvents other than water and relates these phenomena to the surface tension of the solutions in question. He describes tanning as a polar adsorption and considers that it is due to the concentration of the colloidal tannin particles at the collagen–water interface as a result of polar charges. R. M. Lollar,[60] continuing the work of E. E. Doherty[61] on the uptake of tannins by hide powder, also interprets his results as indicating that the uptake of quebracho is "sorptive" in nature. This term is used to include the possibilities of both adsorption and absorption. The work of P. C. Chang, C. T. Yen, and I. L. Yoh[62] on the effect of concentration on the fixation of soluble matter from valonia extracts by hide powder is also in agreement with the "sorption" theory of tanning.

It is difficult to say, however, where adsorption of tannin ceases and chemical combination begins. The effect of modification of the active groups of collagen on the fixation of tannin, for instance by deamination, is perhaps more plausibly explained on the basis of chemical combination than by adsorption, although change in the number of positively or negatively charged groups in the collagen molecule is also likely to affect adsorption. It is also doubtful whether adsorption alone can account for the comparatively high thermal stability of vegetable-tanned leather and a number of workers[55, 63, 64] are of the opinion that this stability is due to the formation of cross-links between adjacent polypeptide chains by means of the tannin molecules.

E. R. Theis and W. H. Blum[63] have studied the effect of various vegetable tannins and other organic compounds on the shrinkage temperature of collagen. On the basis of the observation that the vegetable tannins and benzoquinone increase the shrinkage temperature whilst phenol does not, they conclude that increase in thermal stability is due to the formation of cross-links between adjacent polypeptide chains. Phenol, being capable of reaction with only one active group of collagen, is not able to form such links. Benzoquinone, on the other hand, with which there is the possibility of combination with two active groups thus linking two polypeptide chains together, caused a marked increase in thermal stability. J. L. Stoves[65] also finds that benzoquinone greatly increases the resistance of keratin fibres to stretching. From the effect at different pH values he concludes that the increased stability involves the formation of cross-links between amino-groups in adjacent polypeptide chains as well as rebuilding of the disulphide link.

Whatever the mechanism involved, the fixation of tannin by hide is dependent on the accessibility of the protein to the tannin. R. O. Page[66]

[60] *J. Amer. Leather Chem. Assoc.*, 1943, **38**, 51; B., 1943, II, 267.
[61] P. C. Chang and E. E. Doherty, *ibid.*, 1941, **36**, 160; B., 1941, II, 243. E. E. Doherty and W. Retzsch, *ibid.*, 442; B., 1941, II, 447.
[62] *J. Chinese Chem. Soc.*, 1942, **9**, 82; B., 1943, II, 300.
[63] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 553; B., 1943, II, 137.
[64] J. A. Wilson and I. H. Porth, *ibid.*, 1943, **38**, 20; B., 1943, II, 232.
[65] *Trans. Faraday Soc.*, 1943, **39**, 301; B., 1944, II, 37.
[66] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 116; B., 1943, II, 301.

is of the opinion that the distribution of tannin in vegetable-tanned leather is dependent on the degree of swelling ; the greater is the swelling the more easily can the tannin molecules penetrate. He finds that if the tan is allowed to penetrate through about one third of the thickness, the hide then swollen in acid, and the tannage completed, there is more tannin fixed in the inner than the outer layers. The use of acetone solutions of tannin in the early stages, to facilitate the penetration of tan, has been suggested by W. T. Roddy.[67]

A. Cheshire and N. L. Holmes[68] have studied the equilibrium in leather–water systems at various concentrations. The results obtained suggested that all the water in the systems was not free to act as solvent. The bound water was determined by a number of methods and a probable value of about 50% on the hide substance was obtained. H. C. Holland,[69] however, considers that moisture is also bound by the tanning material. He has studied the effect of increasing degree of tannage on the moisture content of various leathers and apportions this moisture to hide substance, water-solubles, and fixed tan on the basis of the moisture held by these separately under the same conditions. He points out that if the moisture content is calculated on a hide substance basis the improbable result of increasing hydration with increasing degree of tannage is obtained. On his interpretation of the distribution of the moisture, however, the hydration of the hide substance decreases with the degree of tannage in vegetable-tanned leathers except in the case of mimosa tannage. With chrome leather, tannage with chromium salts did not affect the amount of moisture associated with hide substance.

Mineral Tanning.

Little work has been published on this subject during the past year. E. Elöd[70] has continued his controversy with A. Küntzel on the interpretation of the light-absorption of chromium salts solutions in the presence of amino-acids and gelatin.

G. D. McLaughlin and R. S. Adams[71] have published a further paper on the mechanism of chromium sulphate tannage in which they discuss the effect of various methods of removing the excess of solution from the leather on the amount of chromium and sulphate bound. S. G. Shuttleworth[72] has developed a conductometric titration technique for the elucidation of the structure of complex chromium cations by which he claims that the number and proportion of the acidic and hydroxyl groups

[67] *J. Amer. Leather Chem. Assoc.*, 1943, **38**, 184; B., 1943, II, 267.
[68] *J. Soc. Leather Trades' Chem.*, 1942, **26**, 237; B., 1943, II, 33.
[69] *Ibid.*, 1943, **27**, 207; B., 1943, II, 397.
[70] E. Elöd, T. Schachowskoy, and T. de C. Meyer, *Kolloid-Z.*, 1942, **28**, 341; B., 1942, II, 448.
[71] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 530; B., 1943, II, 137.
[72] *Leather Ind. Res. Inst. (S. Afr.)*, 1942, **2**, 44; *J. Soc. Leather Trades' Chem.*, 1943, **27**, 104; B., 1943, II, 267.

inside and outside the complex can be estimated. He finds that chrome liquors produced by reduction of sodium dichromate with sulphur dioxide are mainly composed of a $33\frac{1}{3}\%$ basic chromium sulphate complex containing non-ionised hydroxyl and sulphate groups. On the addition of alkali a $66\frac{2}{3}\%$ basic complex is formed in which 75% of the hydroxyl and all the sulphate groups are covalently linked to chromium atoms.

The results of investigations on the effect of organic acids on the uptake of chromium have been applied to the practical aspects of leather manufacture.[73]

With regard to the use of metals other than chromium, the possibility of iron–chromium combination tannages has been considered[74] and the use of zirconium salts for tanning has been described[75]. The effect of aluminium salts on the shrinkage temperature of collagen has been examined by E. R. Theis,[76] who considers that although the increases in thermal stability suggests that cross-links are formed between adjacent polypeptide chains by means of the aluminium complex, such links are less stable than the corresponding chromium limks.

The retannage of vegetable-tanned leather with chromium and aluminium salts has received some attention. P. S. Briggs[77] has studied the semichrome process, *i.e.*, the retannage with chromium salts of vegetable-tanned leather from which the tan has been partly stripped with alkali. He finds that the amount of chromium fixed, especially in the first stages of tanning, is dependent on the amount of alkali used. For this reason it is suggested that the chrome liquor should be added gradually in order to avoid drawn grain. The fixation of chromium was also dependent on the basicity of the chrome liquor and the length of time during which the skins were horsed up after tanning.

C. W. Beebe and R. W. Frey[78] have compared the properties of various types of vegetable-tanned leather retanned with aluminium and chromium salts. Retannage increased the permeability to air and the resistance to wetting, and decreased the absorption of water, but did not affect the permeability to water vapour. The shrinkage temperature was raised considerably but the strength, whether measured as tensile strength or resistance to tearing or bursting, was unchanged. The aluminium-retanned leathers were at least equal and in many cases superior to the chrome-retanned leathers in strength and resistance to heat and acids.

[73] J. Burchill, A. S. Callaghan, and R. E. Hunnam, *J. Soc. Leather Trades' Chem.*, 1943, **27**, 83; B., 1943, II, 301.
[74] K. F. Ruppenthal and J. P. Malik, *J. Amer. Leather Chem. Assoc.*, 1943, **38**, 148; B., 1943, II, 301. B. M. Das, B. N. Pal, and M. Chaudhuri, *J. Soc. Leather Trades' Chem.*, 1943, **27**, 119; B., 1943, II, 301.
[75] H. G. Turley and I. C. Somerville, *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 381, 391; B., 1942, II, 448; 1943, **38**, 326.
[76] *Ibid.*, 499; B., 1943, II, 67.
[77] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 2; B., 1943, II, 137.
[78] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 478, 539; B., 1943, II, 33, 137.

Formaldehyde Tanning.

K. H. Gustavson[79] has studied the formaldehyde–collagen reaction in some detail, investigating the fixation of formaldehyde by collagen and deaminated collagen under various conditions and its effect on the stability of collagen as measured by the shrinkage temperature, resistance to tryptic digestion, and swelling. He concludes that increased stability is connected with fixation of formaldehyde by the ϵ-amino-groups of the lysine residues in such a way that cross-links are formed between adjacent polypeptide chains. Although there was an appreciable fixation of formaldehyde by the guanidine groups of arginine, and possibly also by the peptide groups, this did not affect the stability of the collagen. In acid solutions, with high concentrations of formaldehyde, appreciable amounts of formaldehyde were fixed and there was an appreciable increase of stability, depending on the time of treatment. It is suggested that in the presence of large amounts of formaldehyde the equilibrium between charged and uncharged amino-groups is displaced by the reaction of the uncharged groups with formaldehyde so that an appreciable number of such groups eventually combine with formaldehyde.

K. H. Gustavson also finds that comparatively large amounts of formaldehyde combine with vegetable- and chrome-tanned leather, producing a marked increase in thermal stability. The same effect was observed by E. R. Theis and W. A. Blum,[63] who suggest that the formaldehyde either acts as an independent tanning agent forming additional cross-links or causes polymerisation of the tannin molecules giving increased size and, therefore, greater tanning effect.

The reactions of formaldehyde with amino-acids and other proteins are also of interest as they may throw further light on the mechanism of the combination of formaldehyde with collagen. E. H. Frieden, M. S. Dunn, and C. D. Coryell[80] have studied the equilibrium between various amino-acids and formaldehyde, using polarimetric and titration methods; they conclude that in most cases two molecules of formaldehyde are combined with each amino-group. D. C. Carpenter and F. E. Lovelace,[81] using similar methods, conclude that with glutamic and aspartic acids each amino-group reacts initially with one molecule of formaldehyde, followed, when the concentration of formaldehyde is high, by a second molecule. Substituents in the side-chains affected the amount of excess of aldehyde necessary for the second reaction to take place; twice as much excess of aldehyde was needed with glutamic acid as with aspartic acid. It does not follow that the compounds which exist in solution can be isolated, and Carpenter and Lovelace were unable to isolate their diformyl derivatives. A. K. Smith, P. Handler, and J. N. Mrgudich[82] have

[79] *Kolloid-Z.*, 1943, **103**, 43; A., 1944, II, 27.
[80] *J. Physical Chem.*, 1942, **46**, 215; 1943, **47**, 10, 20, 85, 118; A., 1942, I, 204; 1943, I, 181, 201.
[81] *J. Amer. Chem. Soc.*, **65**, 1161; A., 1943, II, 294.
[82] *J. Physical Chem.*, 1940, **44**, 874; A., 1941, II, 60.

prepared the formaldehyde derivatives of lysine, arginine, and histidine with the object of gaining further information on the mode of reaction of formaldehyde with the residues of these amino-acids in the intact protein molecule. Analyses most nearly agree with fixation of three molecules of formaldehyde by each amino-acid and it is suggested that the formaldehyde forms a cross-link between the two basic groups of these amino-acids.

With regard to the reaction of formaldehyde with other proteins it has been suggested that the reaction of formaldehyde with keratin involves combination with cysteine residues as well as with amino-groups.[83] W. R. Middlebrook and H. Phillips,[84] however, are of the opinion that the reaction does not take place at ordinary temperatures, except at pH values above 9·0. R. L. Hegmann[85] also could find no evidence of this reaction. J. L. Stoves[86] has studied the effect of formaldehyde on the resistance of keratin fibres to extension. He considers that stabilising links of various types are formed depending on the pH, and that certain of these involve reaction with cysteine.

Physical Properties.

The importance of the physical as well as the chemical properties of leather is now receiving more recognition.

The Physical Testing Committee of the American Leather Chemists Association[87] has been active in the past year and has considered physical tests for leather covering a wide field. These include revised methods for measuring bursting and tearing strength, resistance to abrasion, indentation, flexural fatigue, and deterioration at high temperatures.

An account of the various properties of leather which have received attention and their dependence on the structure of the original skin has been given by D. Jordan Lloyd.[88] Although tanning can modify, it cannot destroy, the original properties of the skin. Not only are there variations in properties in different parts of the skin, but certain properties may also vary in different directions.[88, 89] J. H. R. Bisschop, A. Gardner, J. Sebba, and S. G. Shuttleworth[90] have studied the relations between the breed of animal and the sole leather produced. The leather from each type of hide (Afrikander, Sussex, Red Poll, and Friesland) had a characteristic fibre structure. With regard to resistance to abrasion, however, there was little variation either with breed or age of the animal.

[83] J. H. Bowes and W. B. Pleass, *J. Soc. Leather Trades' Chem.*, 1939, **23**, 365, 453; B., 1939, 972, 1154.
[84] *Biochem. J.*, 1942, **36**, 294; A., 1942, III, 612.
[85] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 276; B., 1942, II, 350.
[86] *Trans. Faraday Soc.*, 1943, **39**, 294; B., 1944, II, 37.
[87] *J. Amer. Leather Chem. Assoc.*, 1943, **38**, 2, 48, 135, 176, 308, 311, 313; B., 1943, II, 267; 1944, II, 27.
[88] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 246; B., 1944, II, 57.
[89] G. O. Conabere, *ibid.*, 1941, **25**, 298, 305, 319; B., 1941, II, 406; B., 1942, II, 126. L. A. Whitmore, G. V. Downing, and W. B. Hudson, *ibid.*, 1940, **24**, 701; B., 1941, II, 103.
[90] *Ibid.*, 1943, **27**, 94; B., 1943, II, 300.

The possibility of combining water-resistance with permeability to air and water vapour is an important characteristic of leather. Methods which have been devised for measuring these properties are mentioned by D. Jordan Lloyd.[88] M. Maeser[91] has developed a method for measuring the air-permeability of light leather; the data obtained indicate that provided the leather can be kept flat without affecting its permeability, the passage of air through the leather obeys Poiseuille's law.

The density of leather is related to its porosity as it gives an indication of the air spaces in the leather. H. Rose[92] has determined both the true and apparent density of a large number of leathers. He introduces the concept of void numbers, which are values representing the volume of air per unit volume of leather substance. Values for true and apparent density were found to vary widely from skin to skin but the physical characteristics of the leather, such as feel and springiness, could be expressed to a considerable extent in terms of void numbers. In any one skin the true density tended to be constant but the apparent density varied with the location of the sample.

J. R. Kanagy and E. L. Wallace[93] have also studied the significance of the density of leather. They find that apparent density is a more sensitive characteristic of the leather than true density. There was some relationship between the percentage of air space in the leather and its permeability to air and water vapour provided grease or wax was not present in large amounts.

The physical properties of alum- and chrome-retanned leathers have been compared[78] (see page 331); accelerated ageing tests on these leathers show that retannage, especially with aluminium salts, increases the resistance to acid deterioration. J. R. Kanagy and P. E. Tobias,[94] however, find that lace leathers tanned only with aluminium salts are much less resistant to accelerated ageing than chrome or Indian (vegetable-) tanned leathers. G. O. Conabere and E. W. Merry[95] have carried out an exhaustive examination of the chemical and physical characteristics of sole leather and relate the physical properties to certain features of the fibre structure.

The effect of impregnation with oils, greases, and waxes on the properties of vegetable-tanned sole leather has been investigated by R. M. Koppenhoefer and R. P. Hermoso.[96] Impregnation with oils made the leather more resistant to water and to abrasion, the effect being related to the viscosity rather than to the composition of the oil. Impregnation with waxes and greases also increased the resistance to water and to abrasion, but the process of impregnation was more difficult.

[91] *J. Amer. Leather Chem. Assoc.*, 1943, **38**, 3; B., 1943, II, 233.
[92] *Ibid.*, 107; B., 1943, II, 267.
[93] *Ibid.*, 314; B., 1944, II, 27.
[94] *J. Res. Nat. Bur. Stand.*, 1942, **29**, 51; B., 1942, II, 449.
[95] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 39, 59; B., 1943, II, 202, 233.
[96] *J. Amer. Leather Chem. Assoc.*, 1943, **38**, 358; B., 1944, II, 26.

The effect of perspiration on chrome-tanned leather has been investigated by A. Colin-Russ.[97] The resistance to perspiration increased with chromium content and basicity of the chrome complex but decreased with increase in pH.

Analytical Methods.

M. Nierenstein and I. Tryon[98] suggest triglycylglycine as a qualitative reagent for tannins; it yields characteristic precipitates with tannins and tannin-like substances, such as galloyl derivatives of glycol, glycerol, erythritol, and mannitol.

Arising from his work with N. L. Holmes on the equilibrium in leather–water systems,[68] A. Cheshire[99] points out that appreciable errors in the determination of non-tannins may arise in the shake method of tannin analysis, owing to the fact that the water bound by the collagen is not taken into account.

R. O. Page and R. C. Holland[100] have studied the effect of the state of division on the removal of water-soluble material from leather. The total amount of material removed by the Wilson–Kern method of extraction increased with the fineness of subdivision of the samples; the free water-solubles were not affected except that with large pieces longer time was required to reach equilibrium.

A method of investigating the acids and salts in complex mixtures by a conductometric technique has been devised by R. S. Airs and M. P. Balfe[101] and has been applied to the analysis of tan liquors. By suitably controlling the end-points of the titrations it is possible to separate the acids present into three groups according to their strength. The method may be somewhat elaborate for works control but suitable conductometric or potentiometric[102] titration methods for determining the acid and salt content of tan liquors are likely to prove of value in tannery control.

H. G. Turley and F. P. Cronin[103] have continued their work on the analysis of sulphited extracts and have applied their methods to the study of the reaction of bisulphite with ordinary quebracho extract.

[97] *J. Hygiene*, 1943, **43**, 72; B., 1943, II, 233.
[98] *Analyst*, 1942, **67**, 389; B., 1943, II, 98.
[99] *J. Soc. Leather Trades' Chem.*, 1943, **27**, 1; B., 1943, II, 137.
[100] *Ibid.*, 234; B., 1944, II, 26.
[101] *Trans. Faraday Soc.*, 1943, **39**, 102, 107; A., 1943, I, 182.
[102] A. Cheshire, W. B. Brown, and N. L. Holmes, *J. Soc. Leather Trades' Chem.*, 1941, **25**, 254; B., 1941, II, 406.
[103] *J. Amer. Leather Chem. Assoc.*, 1942, **37**, 332, 462; B., 1942, II, 416; 1943 II, 33.