THE SURVIVAL OF HUMAN ERYTHROCYTES

IN VITRO AND IN VIVO

BY

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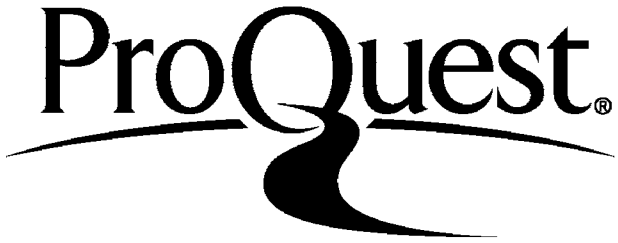
Submitted for the M.Sc. Degree.



Abstract

A quantitative method for determining the number of donor erythrocytes in the blood stream of a recipient is described. Briefly, group O blood is given to a group A recipient; after transfusion two types of cells are present in a sample of the recipient's blood. The recipient's cells are agglutinated with anti-A serum and the free donor cells counted. The 'in vivo' survival of blood, stored for varying times in many recommended and test diluents was determined. A study of the progressive changes in osmotic and mechanical fragilities, degree of haemolysis and pH was made in order to discover whether any correlation existed between 'in vitro' properties and 'in vivo' survival. Judged on the criterion of 'in vivo' survival blood stored without glucose was found to deteriorate after 5 days. Blood stored with glucose lost little of its therapeutic value during 21 days storage. A mixture recommended by Rous and Turner (1916) in which blood is diluted with a large volume of 5.4% glucose was found to be the best diluent, 'in vivo' survival not being impaired until after a month's storage. 'In vitro' tests were found to be no guide to good 'in vivo' survival. Increase in osmotic fragility particularly may lead to most fallacious conclusions. The increased osmotic fragility of cells stored in the Rous-Turner diluent is reversed by plasma or isotomic saline. The

superior survival of the Rous-Turner mixture may be due to the decreased sedimentation rate and therefore slowly so-lecithin formation.

The real value of stored blood depends upon the ability of the transfused erythrocytes to maintain their biological function in the recipient. In spite of the clinical success attending the majority of blood transfusions for the past 25 years, general scepticism prevails regarding the survival of these transfused erythrocytes . Investigators have been more concerned with the immediate safety of a transfusion and the longer term policy has been neglected. However, a wealth of information has accumulated concerning the 'in vitro' behaviour of red cells; many workers predicting 'in vivo' survival from such properties as degree of haemolysis or alteration of osmotic fragility; this has led to the recommendation of a very large number of diluents for the preservation of blood.

The primary object of this investigation was to obtain a method capable of yielding an accurate quantitative figure for the number of donor erythrocytes surviving in the recipient at any time after a transfusion. The method was then used to determine the 'in vivo' survival of blood stored for varying intervals in many of the recommended diluents. Immediately prior to a transfusien many 'in vitro' tests were

performed on the preserved blood to be given in order to discover any correlation that might exist between 'in vivo' survival and 'in vitro' behaviour. It was also hoped that the data obtained might suggest improvements on the present methods of storage.

The 'Inagglutinable Count' Technique and its applications

Peyton Rous and Turner (1916) showed that haemoglobin loss in experimental animals could be replaced by transfused cells, from the same species, stored up to two weeks in a citrate glucose diluent. Rabbits were used and blood counts and blood pigment excretion studied. Ashby (1919) first described a method of following quantitatively the fate of transfused red cells, and all subsequent methods are based on the same principle; group O cells were given to a recipient of group A, so that after transfusion a sample of the recipient's blood contained two types of cells, O and A. The A cells were agglutinated with anti A serum 'in vitro' and the free O cells counted. A fraction of the A cells did not agglutinate and a small 'blank' was therefore subtracted from the total of free cells. Ashby found that fresh blood could be detected in the recipient's blood stream up to 100 days after the transfusion. Wearn et al (1922) and Jervell (1924) confirmed these results. The same figure was also obtained by Wiener (1934) who adopted the method using the subgroups M and N since he preferred to give

identical group transfusions. Wiener and Schaefer (1939, 1940) extended the method to the study of the survival of stored blood.

Since this is the only method available, permitting a direct measure of cell survival and since 100 days is rather a revolutionary figure for the life of the red blood cell, it is profitable to consider the criticisms levelled against the technique. Isaacs (1924) objected firstly, that the inagglutinable 'blank' consisted almost entirely of immature red cells; and secondly, that even fresh blood is rapidly destroyed in the recipient and stimulates the appearance of immature, and therefore inagglutinable, red cells in the blood stream. His evidence is not good, and the following considerations show that the method is valid and reliable.

(1) The percentage of reticulocytes has always approximately the same value in whole blood, the 'blank', and free cell count after any transfusion.

(2) The inagglutinable count should be higher after a transfusion of old blood, than after one of fresh blood, if cell destruction stimulates the liberation of reticulocytes into the blood stream. Actually the number of free cells found after a transfusion is directly proportional to the volume of blood given and inversely proportional tonthe body weight of the recipient. Old blood gives a rise in the in-

agglutinable count which is lower than the theoretical figure due to the destruction of a certain number of the cells during the transfusion.

(3) There is never any rise in the free cell count when group A blood is given to a group A recipient, proving that even if some of the cells are destroyed no cells incapable of being agglutinated are thrown into the blood stream.

(4) Further proof may be obtained fromnthis experiment; group M blood is given to a group N recipient. Agglutination with anti N serum after the transfusion will leave free a large number of cells, but if the recipient's blood is treated with anti N and M sera very few cells will remain free, both recipient and donor cells having been agglutinated.

Technique

Ashby originally used a 1/22 dilution of blood in the appropriate anti-serum; red cell counting at this dilution is very difficult and rouleaux formation common, so that 1/100 dilution was chosen. Her choice of temperature, 37°C, was difficult to understand because agglutination is more complete at lower temperatures; the ice box is unreliable on account of the action of cold agglutinins, so that room temterature is preferable. Forty minutes was found to be an inadequate time for agglutination, therefore, 2 hours was allowed as suggested by Taylor (1938). The following modified technique was used:

20 c.m.m. of recipient's blood was delivered from a haemoglobin pipette into 1 cc 3% sodium citrate. 0.1 cc of this 1/50 suspension was mixed with 0.1cc of the appropriate anti-serum in a small flat bottomed bottle. (The mixture formed a very thin film on the bottom of the bottle, thus sedimentation and agglutination were rapid). The corked bottle was left 2 hours at room temperature and shaken occasionally to ensure complete agglutination. The contents were then mixed thoroughly and a drop placed in a Bürker counting chamber. This chamber was chosen because the large area of ruling enabled a selection of columns to be made when large agglutinates obscured part of the field. All experiments were performed in duplicate.

Both the A B O and M and N groupings were made use of, and this microtechnique was chosen because large stocks of reliable anti M and N sera are not available. The consistency of results depends entirely upon the potency of the sera used, titres of 1/512 at least being required. Excessively high titres are not advisable since these sera nearly always contain haemolysins as well; infected sera also develop haemolytic properties.

The method was found to have the same error as that of ordinary blood counting. [Mollison & Young, 1940. See appendix]. Görl (1926) thought that shaking agglutinates increased the number of free cells; this is very unlikely

since the attractive force between agglutinated cells is very great, and vigorous shaking for 1 min. was not found to increase the free cell count. Finally, there may be some difficulty in determining when two cells are agglutinated or merely stuck together; distortion and discolouration of cells indicates agglutination.

Blood used

Blood was taken from donors, presumed to be healthy, into two diluents:-

a) 1.05% sodium citrate in 0.85% NaCl. 360 cc blood was collected in 180 cc diluent containing sufficient glucose to give a final concentration of 1% in blood diluent mixture.

b) 3% sodium citrate. 440 cc blood being collected in 100 cc diluent with sufficient glucose to give a final concentration of 0.6%.

The blood was stored at 4°C in 540 cc bottles, and given to the recipient as a concentrated cell suspension. This is easily done as the cells soon collect in the bottom of the bottle, and the supernatant plasma-diluent mixture may be removed just before the transfusion. Counts were not made on the citrate saline bottles, but the average figure for the citrate cells given was 6.6 million/c.m.m. 475 cc cells was the average volume given.

The transfusion of a concentrated cell suspension has three advantages over the transfusion of whole blood diluent mixtures. Firstly, it does away with the variations in proportions of blood and preserving mixture, so that a similar volume of cells may be given to each patient and standardised blood volume changes will be produced. Secondly, overloading of the circulation is avoided, a much larger oxygen carrying power being delivered to the patient in a smaller volume transfusion. Thirdly, the transfusion of high titre agglutinins is reduced. This is particularly important since it has been shown recently by Aubert (1942) that considerable destruction of recipient cells may occur, the theoretical dilution of donor agglutinins not taking place before some agglutinin at least is absorbed by the recipient's cells.

Clinical Material

The majority of patients suffered from secondary anaemia connected with pregnancy or parturition. Any with haemolytic anaemia, sepsis or intracurrent haemorrhage were rejected.

Results

These are expressed graphically in Figs. 1 and 2. The citrate saline glucose figures represent a preliminary investigation. Samples of blood were taken from the patients immediately before the transfusion, afterwards at varying intervals up

to 24 hours, and then at convenience during 3 months. The figures obtained were partially standardised by being divided by the number of bottles of blood given.

The citrate glucose results are expressed differently from those above, but in the same manner as the results in the next section. Specimens of blood were collected from the recipients immediately before transfusion; the transfusions took from 60 to 120 mins. and other samples were collected 5 mins. after the transfusion, 4 - 8 hours afterwards, 24 hrs. afterwards and then at convenient intervals.

When blood stored0-4 days was transfused the 4-8 hr. sample showed a rise in the free cell count. With older bloods this second figure was equal to, or smaller than, the first obtained after transfusion. It would be ideal to take the 100% figure as the number of cells present when all blood volume readjustments had taken place, i.e. 48 hrs. after the transfusion. In all but very fresh blood transfusion there would be destruction during that period, so it was decided to express all figures as a percentage of the figure obtained immediately after transfusion, although the 4-8 hrs. and 24 hrs. samples for fresh blood then appear greater than 100%. In many old bloods some destruction must actually occur during the transfusion; this is a further source of error. The blood volume of the patients could have been estimated from a

knowledge of their height and weight, and the theoretical rise in free cell count calculated and taken as the 100% value. However, as many of the patients were anaemic or pregnant and there are definite but unstandardised blood volume changes associated with these conditions, this was not ~~carried out~~ calculated. It is seen (Fig.1) that the values for survival lie upon an even slope which is very similar for all blood stored up to 18 days. The total survival of this age group is approximately 100 days which agrees well with Ashby's figure for fresh blood. The older blood is destroyed more rapidly.

Exactly similar results were obtained with blood stored in the citrate-glucose diluent. The individual survivals in each group chosen showed close agreement with one another. The 0-4 days group shows the 104% figure for 24 hours and a total survival of at least 100 days. Destruction is more rapid initially in the 11-15 day group, but the total time of survival is little inferior to that of fresh blood. With the older bloods the period of most rapid destruction is during the first 24 hours after transfusion and this is borne out by the serum bilirubin rises which also occur; total survival is shorter, and gradually decreases with increasing age of blood. The slopes shown in Fig. 2 are taken as standards for comparison in the next section.

IN VIVO SURVIVAL

FIG. 1.

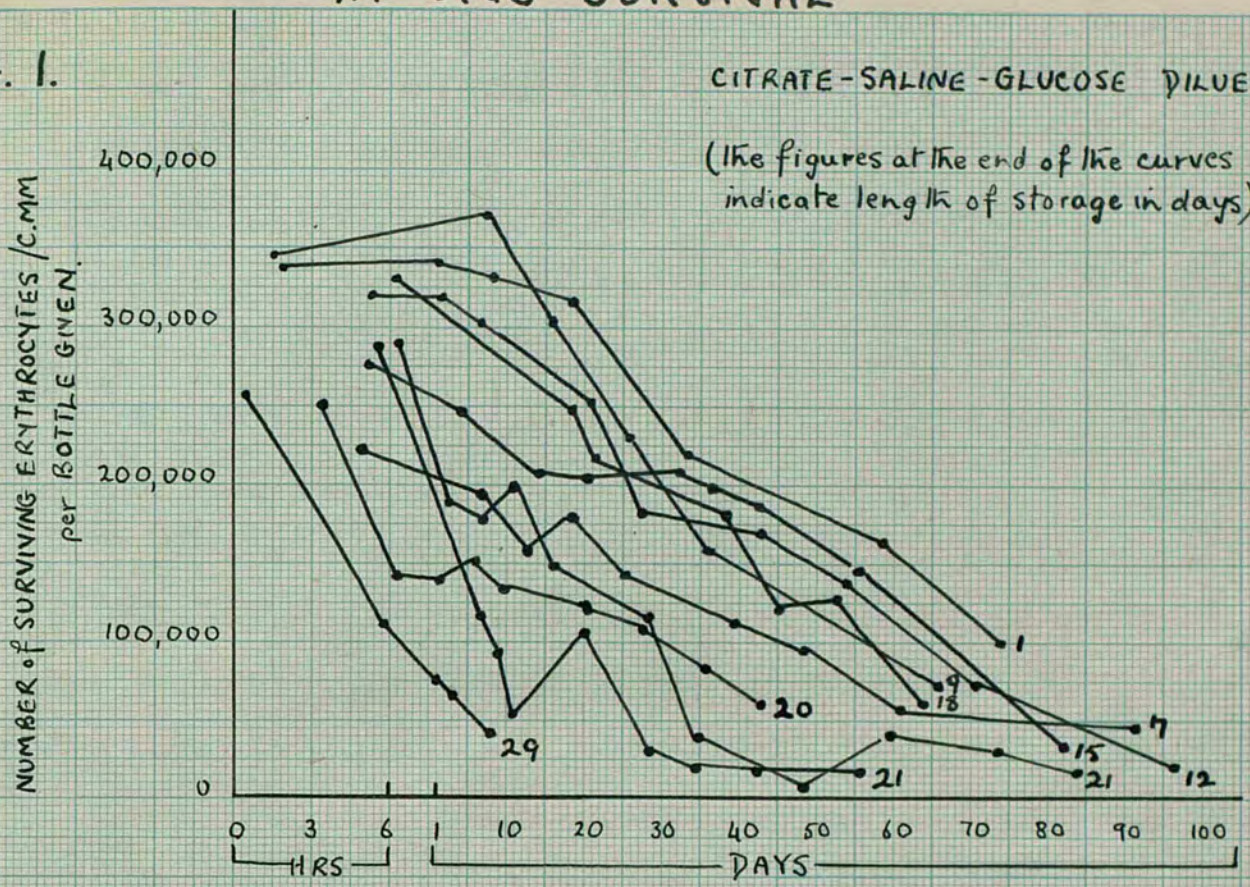
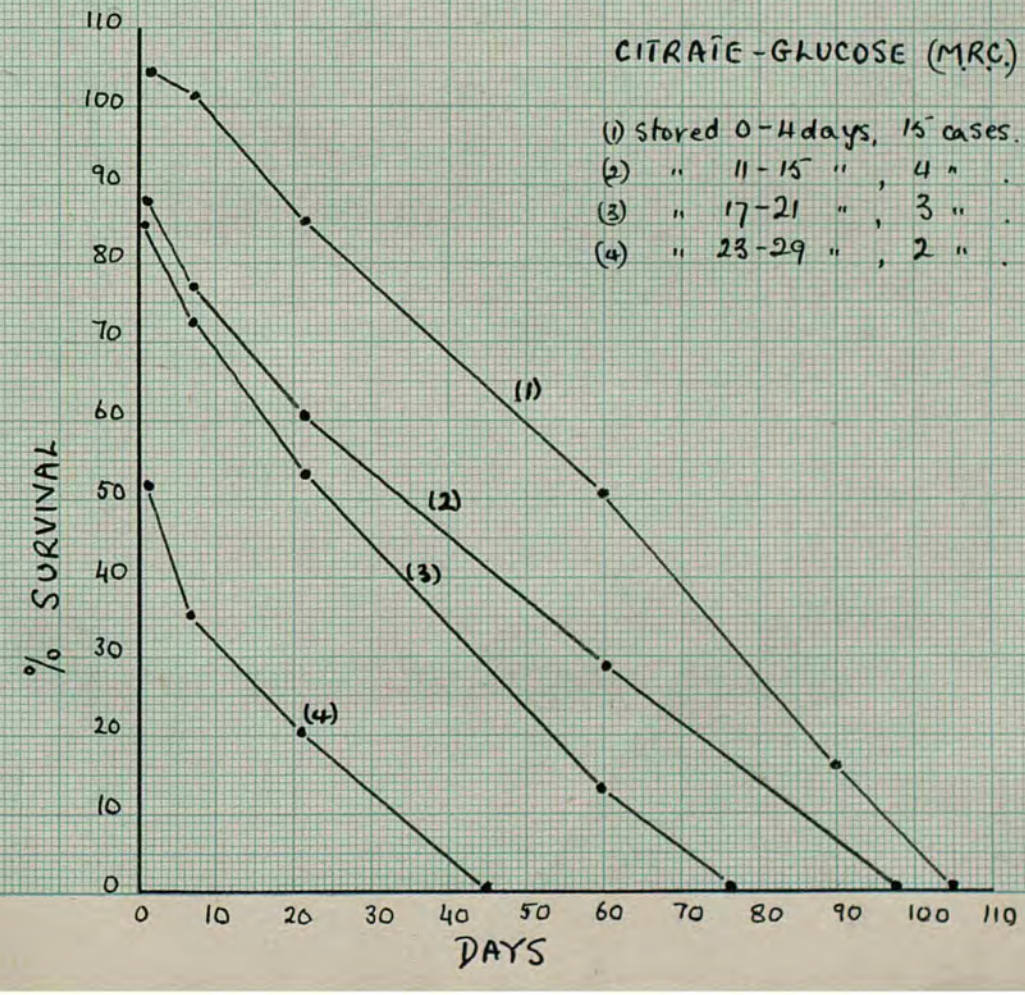


FIG. 2.



The investigations of Bushby et al (1940), Maizels and Patterson (1940), Belk and Barnes (1941), all confirm these results, and it would be reasonable to generalize that blood in the Medical Research Council's citrate glucose diluent loses little of its therapeutic value during 21 days storage, but thereafter deterioration is rapid.

In Figs. 3-8 are shown the results of two simultaneous transfusions in the same patient using the A B O and M and N agglutinating systems together. Bloods of two different ages were compared, or blood stored in two different diluents. These experiments are in the nature of controls, since the two bloods are compared under exactly the same conditions. The results show clearly that the more rapid destruction of older blood is due to deterioration of the red cells and not to any idiosyncrasy of a patient. Two other controls are shown in Fig. 9. The recipient and donor grouping was such that the recipient's cells could be agglutinated by two different antisera; the results were found to agree closely.

Blood volume

This cell agglutination technique has many other applications, especially in the realm of pathology; blood destruction may be studied in cases of pernicious anaemia, sepsis, acholuric jaundice and so on. It may also be used in the determination of blood volume as was suggested by Ashby; the

SURVIVAL OF TWO DIFFERENT TRANSFUSIONS GIVEN SIMULTANEOUSLY TO THE SAME PATIENT.

FIG. 3.

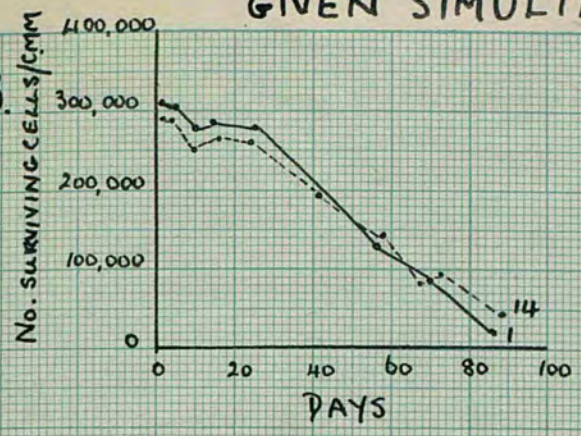
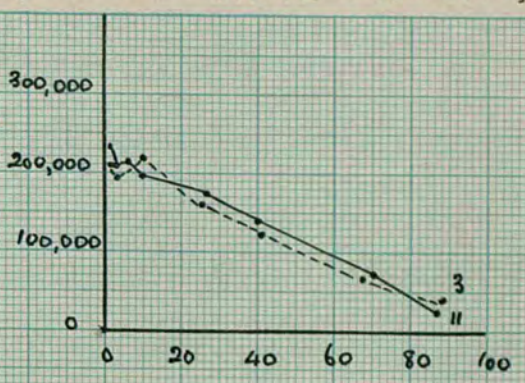


FIG. 4.



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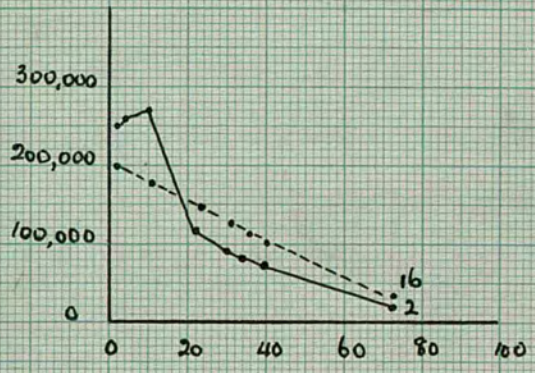

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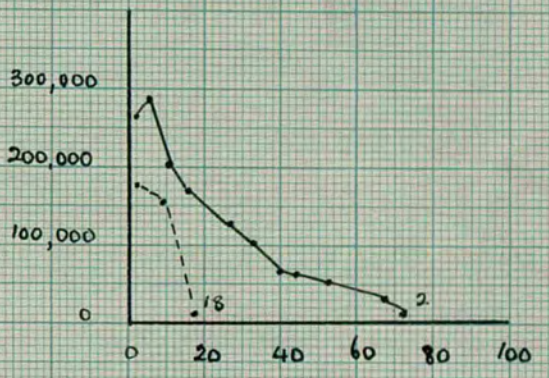

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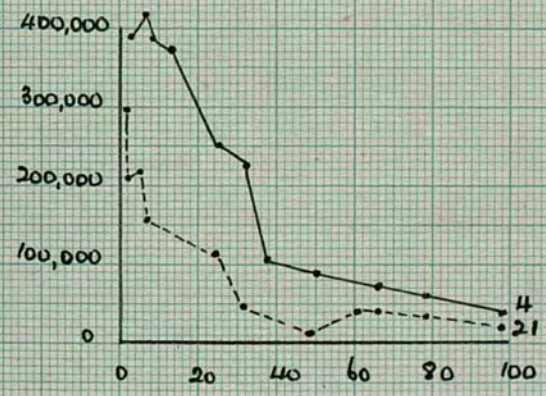
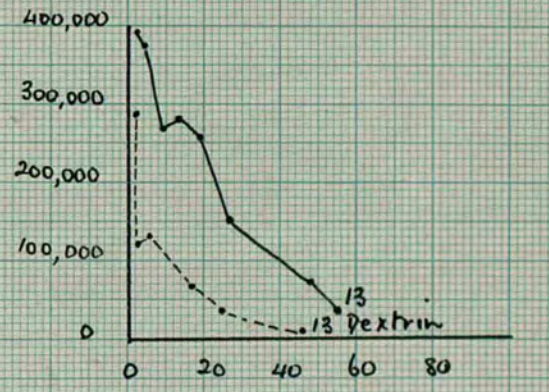


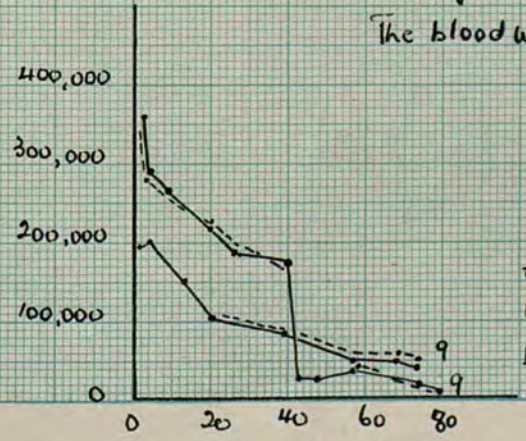
FIG. 8.



(figures at end of curves indicate length of storage in days)

The blood was stored in Citrate-Saline-Glucose diluent.

FIG. 9.



Note (Fig. 9.)
Survival of two single transfusions given to different patients. The continuous lines show figure found when agglutination performed with sera of ABO system; the dotted were obtained with M N sera.

calculation is made from the following equation:

$$\frac{\text{Number cells /c.m.m. blood given}}{\text{Number 'free' cells/c.m.m. recipient's blood after transfusion}} = \frac{\text{Recipient's blood volume}}{\text{volume of blood transfused}}$$

'Free' cells are those left unagglutinated when the recipient's blood is treated with the appropriate anti-serum.

The two obvious advantages of giving blood in place of a dye are: (1) that the red cells can only be removed from the circulation via the reticulo endothelial system; if fresh blood is given this should not occur; (2) no apparatus and reagents, excepting good antisera, are required besides those used in ordinary blood counting. The great disadvantage however is that a fairly large bulk of fluid is added to the circulation and the true blood volume figure cannot be obtained until the transfused plasma, and any of the recipient's plasma (to compensate for the increased volume of corpuscles present) have been removed from the blood stream. Assuming no cells are destroyed, then samples of the recipient's blood could be tested over a period of 0-72 hours after the transfusion until a constant figure was obtained.

A careful study of blood volume was not made, but blood volumes were calculated in a few cases which received fresh blood, the free cell count immediately after transfusion being used so that no circulatory readjustment had taken place. The results are shown in table I; the volume of blood given has

TABLE I

Blood volume estimations

	Patient	Blood Volume: Determined from height and weight in litres	Blood Volume: Found	Patients' Haemoglobin before transfusion % Haldane standard	Volume of blood given in cc.
Men	1	5.0 (approx)	7.3) 6.9)	102	990 blood
	2	5.0	4.7	100	900 cells
	3	5.0 (approx)	5.7	100	650 cells
Women	4	4.2	5.0	64	520 cells
	5	4.0 (approx)	4.5	58	1,050 blood
	6	4.0 (approx)	5.4	58	980 cells
	7	4.0	3.0) 3.4)	52	1,040 blood
	8	4.0 (approx)	3.7	50	410 cells
	9	4.2	4.0) 3.9)	48	635 cells
	10	4.2	4.2	48	1,040 blood
	11	3.7	3.3) 3.0)	42	750 cells
	12	4.15	4.0) 3.7)	40	745 cells
	13	3.7	3.1	35	450 cells
	14	4.0	2.9) 3.0)	25	1,000 blood
Pregnant women	15	4.0	4.8	86	435 cells
	16	4.0	5.0	72	400 cells
	17	3.9	3.9	71	525 cells
	18	3.6	6.0	61	310 cells
	19	4.0	5.8	46	420 cells

Where two figures are given the values were calculated from samples of recipients blood at two different times after the transfusion.

not been subtracted from the estimated blood volume. Knowing the patient's height and weight, the theoretical blood volumes were obtained from the curves given by Gibson and Evans (1938).

Pregnant women have increased blood volumes due to an increased plasma volume, and anaemic patients usually have a reduced blood volume due to reduction in both plasma and red cell volume. (Gibson 1941). The results obtained are in substantial agreement with these observations.

Investigation of the correlation between 'in vivo' survival and 'in vitro' behaviour

Erythrocyte deterioration most probably depends upon degeneration of the red cell membrane which must certainly be postponed by maintaining the normal nutrition and metabolism of the cell. (Most investigators have shown a small but significant oxygen consumption by the erythrocytes. Ponder 1934). The osmotic relationships between cell and plasma-diluent mixture seem to be related to membrane degeneration indirectly through the mechanical effects of stretching or shrinking.

Rous and Turner (1916), Maizels and Whittaker (1939) and de Gowin and Hardin (1940), concluded that a preservative was good if it inhibited haemolysis during storage. Other workers, Wilbrandt (1940), Bushby et al (1940), Willeneger and Ottensooser (1940), Dubash et al (1940), Denstedt et al (1941), and Crosbie and Scarborough (1942) preferred

that their diluents should inhibit increase of osmotic fragility.

The substances which have been found to inhibit haemolysis and increase in osmotic fragility are:
a) certain electrolytes, b) glucose, which diffuses rapidly into the red cell and c) non diffusible substances such as sucrose and dextrin, which oppose the osmotic pressure due to haemoglobin. Bagdassarov (1937) found that a citrate saline mixture containing potassium chloride and magnesium sulphate delayed haemolysis better than plain citrate; this solution, known as I.H.T. on account of its use at the Institute of Haematology and Blood Transfusion in Moscow, has been widely used in England. Glucose was first shown by Rous and Turner to delay haemolysis due to mechanical trauma and since then many of the investigators, quoted above, have found that it is very efficient in inhibiting haemolysis and increase in osmotic fragility in stored blood. Much attention has been paid to the concentration required, but small amounts only seem to be necessary, the maximum effect showing when a final concentration of 1% is present in the blood-diluent mixtures. Wilbrandt recommended the use of sucrose in a final concentration of 4.3% because he found that the fragility of the cells remained within normal limits for 100 days. Maizels had the idea of using dextrin which was non-diffusible, but which at the same

time might form a reservoir from which dextrose would slowly be liberated. Haemolysis was only 1% after 50 days storage in a final concentration of 3% dextrin.

Blood preserved in the diluents recommended separately by Rous and Turner and by Wilbrandt were first compared 'in vivo'. The survival of Rous-Turner cells was very good, the degree of haemolysis was low but osmotic fragility very high, the median corpuscular fragility being shown in 0.75% NaCl after 21 days storage. Cells stored in Wilbrandt's sucrose mixture survived poorly, haemolysis was low, and median corpuscular fragility was shown in solutions well below the average normal figure of 0.36% NaCl. The striking difference between these results led to the choice of many of the diluents described in Tables II and III. Defibrinated and heparinised bloods served as controls since nothing was added to them. Citrate (III), I.H.T.(IV), M.R.C. citrate glucose (VI), Rous Turner citrate glucose (VIII), Maizel's dextrin(XII) and Wilbrandt's sucrose were all investigated because they had been recommended from the result of in vitro tests. Diluent V represents the large dilution of plasma proteins in the Rous-Turner mixture but lacks glucose, XI is similar but contains glucose equivalent to that in the M.R.C. citrate-glucose diluent. VII reproduces the high glucose concentration of Rous-Turner without the dilution. IX and X were an attempt to produce the Rous-Turner conditions on a smaller scale.

TABLE II

List of Preservatives used

1. Defibrinated blood
   (Harrison & Picken)
   30 cc glass beads
   450 cc blood.

2. Heparinised Blood
   10 cc saline containing
   Liquemin 15,000 A.C.U.
   or B.D.H. Heparin
   2000 units.
   450 cc blood.

3. Sodium Citrate
   100 cc 3% sodium citrate
   440 cc blood

4. I.H.T.
   Equal parts of blood
   and mixture containing
   0.7% NaCl
   0.52% sodium citrate
   0.02% KCl.
   0.004% $MgSO_4$

5. Sodium Citrate
   1,400 cc 3% sodium citrate
   600 cc blood.

6. Citrate glucose (M.R.C.)
   100 cc 3% sodium Citrate
   10 cc 30% glucose
   430 cc blood.

7. Citrate glucose
   100 cc. 3% sodium citrate
   40 cc 30% glucose
   400 cc. blood.

8. Citrate glucose (Rous Turner 1916)
   400 cc 3.8% sodium citrate
   1,000 cc 5.4% glucose
   600 cc blood.

9. Citrate glucose
   100 cc 3% sodium citrate
   100 cc 5.4% glucose
   340 cc. blood

10. Citrate glucose
    40 cc. 5% citrate
    180 cc. 5.4% glucose
    320 cc. blood

11. Citrate glucose
    1,400 cc 3% sodium citrate
    40 cc 30% glucose
    560 cc blood.

12. Dextrin (Maizels 1940)
    100 cc 3% sodium citrate
    30 cc 53% dextrin
    410 cc blood

13. Sucrose (Wilbrandt 1940)
    63 cc 10% sodium citrate
    375 cc 10.3% sucrose
    410 cc blood.

A sample of the blood transfused was always tested for percentage haemolysis, osmotic fragility and mechanical fragility, the latter test was chosen with the idea that it might give some indication of cell membrane degeneration. In addition a separate in vitro investigation was carried out so that progressive changes in each diluent could be studied over four weeks. Blood from 3-4 donors was used for each diluent. To prevent the bottles becoming infected through frequent opening each was partitioned,under sterile conditions, into about 12 smaller bottles, immediately the blood was collected. An undisturbed bottle was therefore available for tests at any time. pH estimations were also made in this series because little information is available of the changes in pH which take place in stored blood.

Methods

The clinical material, concentrated cell suspensions given and methods of sampling and inagglutinable count were all described in the last section.

The blood tested for 'in vitro' properties was either diluted with some of its own supernatant liquid or spun, and some of the supernatant liquid removed, so that the final haemoglobin was always 70% of the Haldane standard. This standardisation enabled a haematocrite reading of 30% to be assumed in the estimation of spontaneous haemolysis;

furthermore the same volume of cells was always added to each 1 cc of saline in the determination of osmotic fragility.

Spontaneous Haemolysis

The supernatant plasma diluent from well mixed and centrifuged blood was matched with dilute haemoglobin standards ranging from 5% to 0.1% (Haldane). The standards were prepared from a small quantity of blood, of known haemoglobin content, laked in a little water and diluted with serum or plasma.

Percentage haemolysis =

$$\frac{\text{total quantity Hb in plasma of 100 cc blood}}{\text{\% Hb of the blood sample}} \times 100\% .$$

Since haemoglobin of blood was made to be 70% and plasma volume 70%, the percentage haemolysis is equal to the haemoglobin standard matched by the unknown.

Osmotic Fragility

The method of Dacie and Vaughan (1938) was used, the strength of NaCl in which (1) 50% haemolysis (M.C.F or median corpuscular fragility) and (2) trace haemolysis occurred were recorded only. Fragility was estimated at room temperature after it was found that the values differed insignificantly from those obtained at ice box temperature.

Mechanical Fragility

Rous and Turner estimated mechanical haemolysis but published no method. Blood shaken in a mechanical shaker for several hours shows no measurable degree of haemolysis. Glass beads were therefore added in varying proportions until it was found that haemolysis in 4 week old blood was five times that obtained for fresh blood.

5 cc of blood and 10 cc glass beads (average diameter 0.4 cm.) were delivered into a 10 oz. flat bottle which was shaken horizontally on its narrow side for exactly ½ hr. in a mechanical shaker. The blood was centrifuged, the plasma haemoglobin estimated and percentage haemolysis calculated.

pH Determinations

Dr. Hewitt of the Belmont Laboratories Sutton, Surrey, very kindly made the pH determinations, the results of which are given in Fig.19 The glass electrode method was used.

Results

'In vivo' Survival is shown in Figs.10-15 and table III. Fig.10 demonstrates the various types of initial changes which are encountered, varying from the rise already discussed for fresh blood, to the rapid destruction of useless blood.

Blood stored without glucose does not preserve well, rapid deterioration taking place after 5 days storage. This

FIG. 10

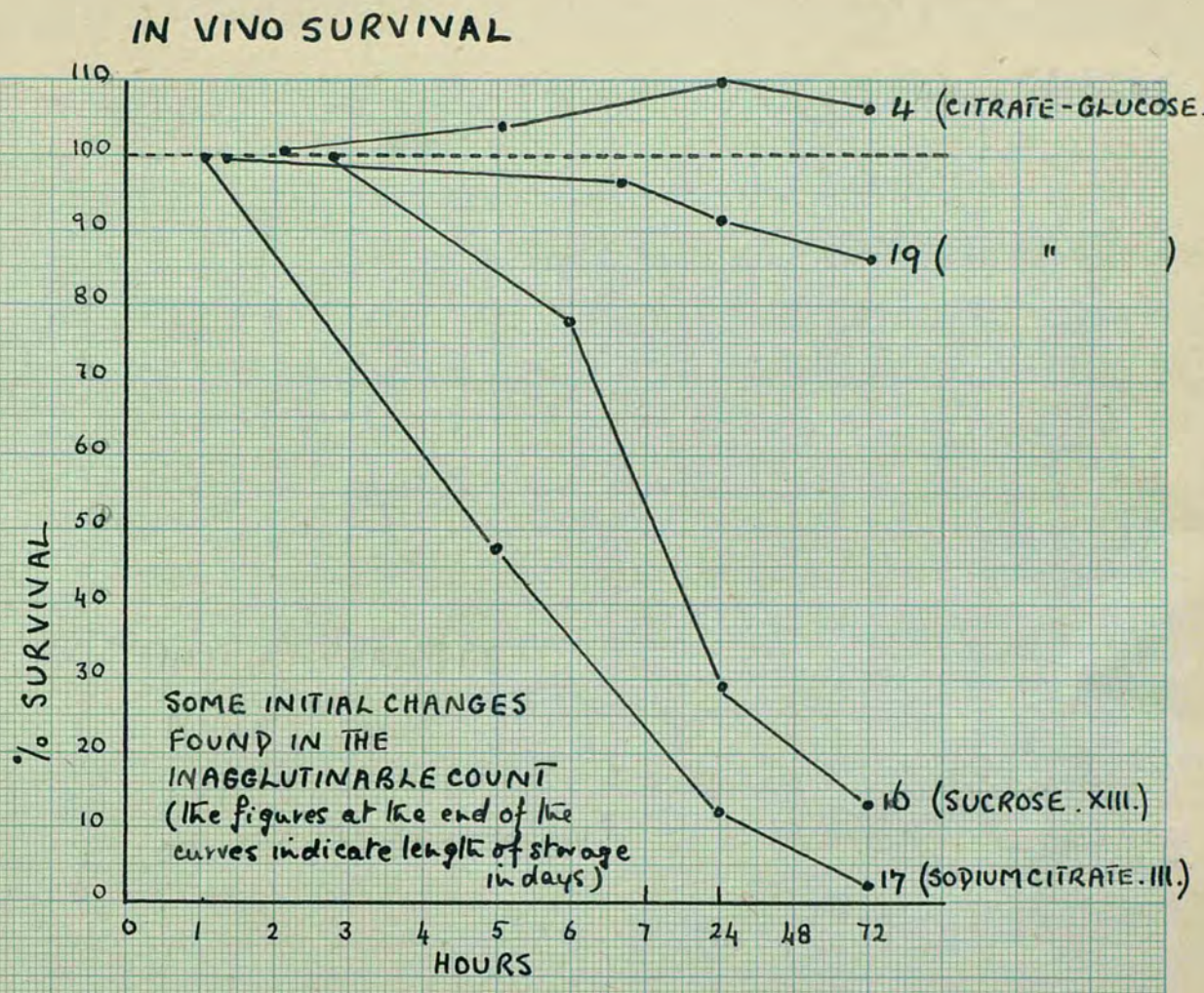

IN VIVO SURVIVAL
% SURVIVAL
110
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 24 48 72
HOURS
4 (CITRATE-GLUCOSE.VI.)
19 (")
16 (SUCROSE. XIII.)
17 (SODIUM CITRATE. III.)
SOME INITIAL CHANGES FOUND IN THE INAGGLUTINABLE COUNT
(The figures at the end of the curves indicate length of storage in days)

FIG. 11.

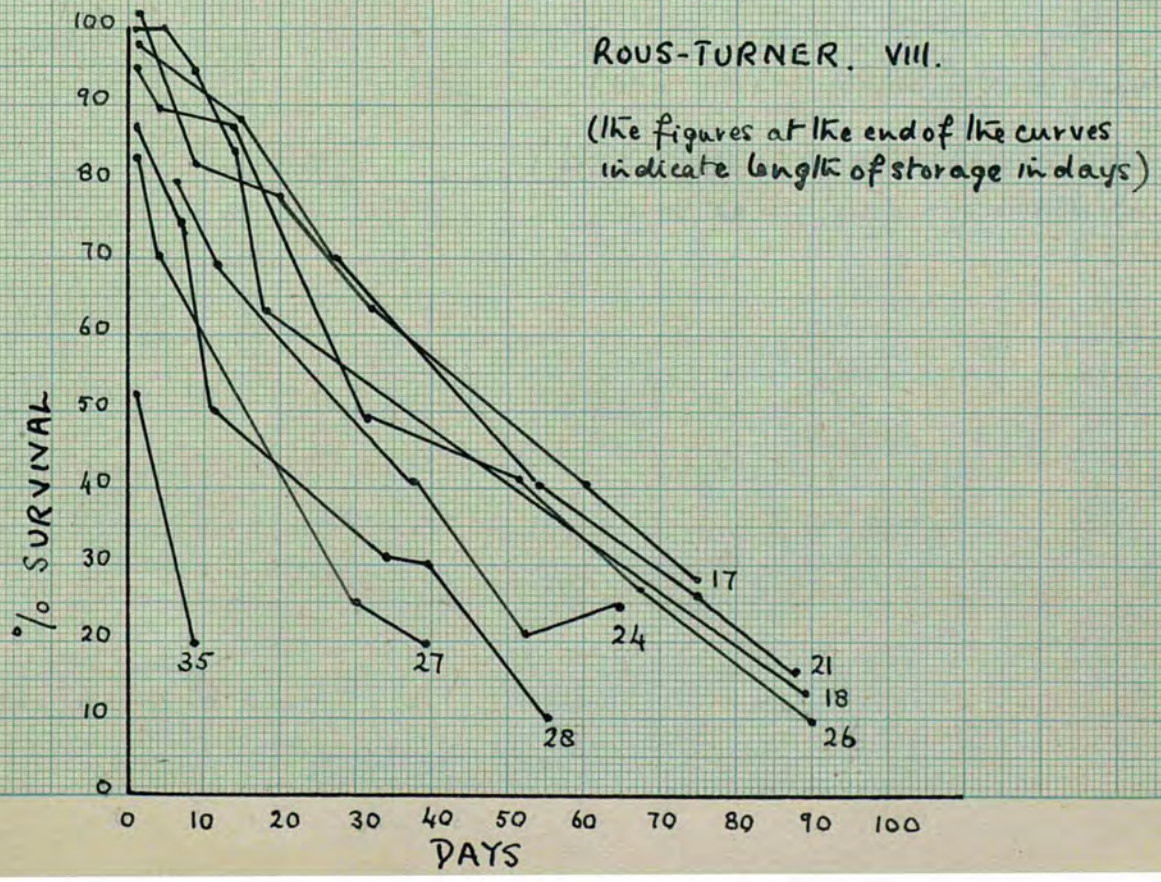

% SURVIVAL
100
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90 100
DAYS
ROUS-TURNER. VIII.
(The figures at the end of the curves indicate length of storage in days)
17
35
27
24
21
18
28
26

FIG. 12.

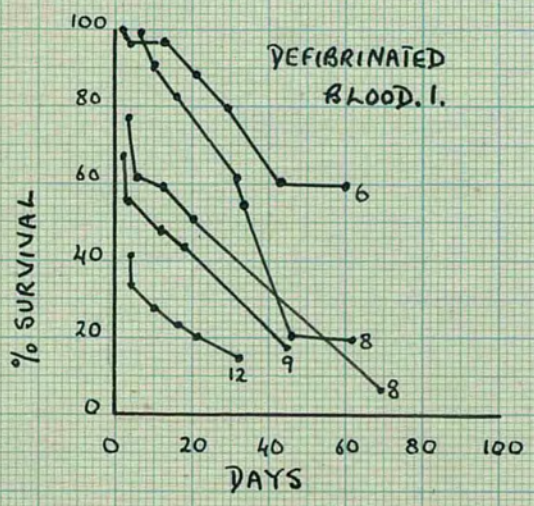


FIG. 13.

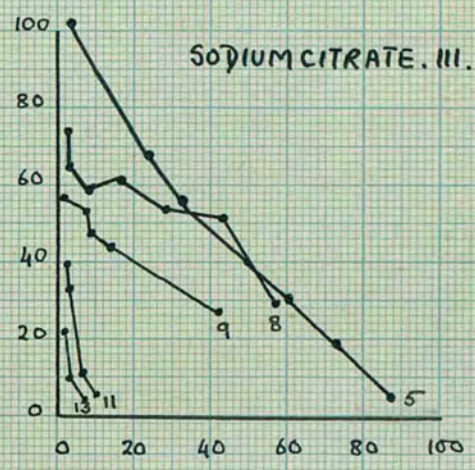


FIG. 14.

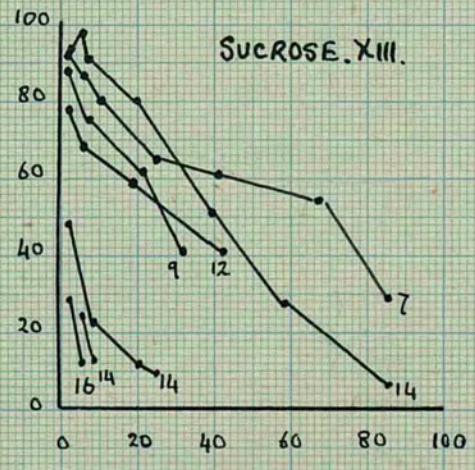
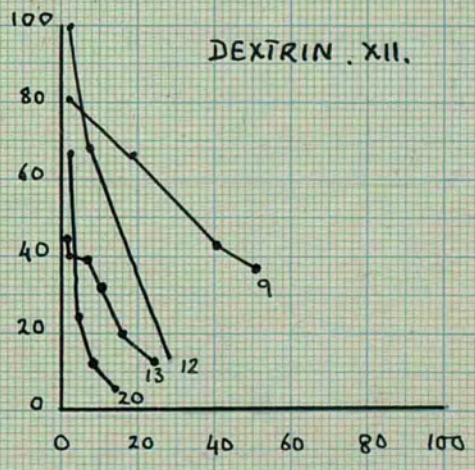


FIG. 15.



IN VIVO SURVIVAL OF BLOOD STORED IN VARIOUS DILUENTS

(The figures at the ends of the curves indicate length of storage in days)

TABLE III

SURVIVAL IN VIVO

	Preservative	Time of Storage in days	No. of cases	% Survival in Vivo			
				24 hrs	1 week	3 weeks	2 months
	Diluents containing no carbohydrate						
I	Defibrinated Blood	6-9	4	89	76	63	20
II	Heparinised Blood	6-10	4	76	61	40	6
III	Sodium Citrate	5-9	3	72	68	51	27
	1 vol : 4·4 vols blood	11-17	3	25	11	-	-
IV	I.H.T. 1 vol : 1 vol blood	8-10	4	78	52	42	20
V	Sodium Citrate						
	7 vols : 3 vols blood	9	2	40	22	10	-
	Diluents containing carbohydrate						
VI	Citrate glucose (M.R.C.)	0-4	15	104	101	86	49
	1·1 vols : 4·3 vols blood	11-15	4	88	77	60	27
	Final glucose 0·6%	18-19	3	84	73	53	13
		23-29	2	52	35	20	-
VII	Citrate glucose						
	1·4 vols : 4 vols blood	11-14	2	89	70	60	24
	Final glucose 2·4%	16-21	4	77	65	48	20
VIII	Citrate glucose Rous-Turner	17-21	3	100	93	74	37
	7 vols : 3 vols blood	24-28	4	83	78	50	12
	Final glucose 2·7%	34-35	2	54	31	-	-
IX	Citrate glucose						
	2 vols : 3·4 vols blood	20	2	76	61	50	17
	Final glucose 1%						
X	Citrate glucose						
	2·2 vols : 3·2 vols blood	18-20	3	88	82	69	19
	Final glucose 1·8%						
XI	Citrate glucose						
	7·2 vols : 2·8 vols blood	16-19	2	53	40	29	12
	Final glucose 0·6%						
XII	Dextrin Maizels						
	1·3 vols : 4·1 vols blood	9	1	82	76	62	30
	Final dextrin 3%	12-13	2	86	57	31	-
XIII	Sucrose Wittbrandt						
	4·4 vols : 4·1 vols blood	7-9	2	85	76	66	34
	Final sucrose 4·3%	12-16	6	55	33	25	8

was also found by Wiener and Schaefer (1939). Defibrinated blood showed the best survival in this group, and the poorest diluent was that containing the large excess of sodium citrate (V).

Blood stored in dextrin and sucrose shows a slight improvement over defibrinated blood. The presence of glucose is most beneficial; a final concentration of 0.6% seems just as efficient as 2.4%, and deterioration does not begin till the blood has been stored 15 days. The Rous-Turner diluent is superior to all others; deterioration is not evident until 24 days storage.

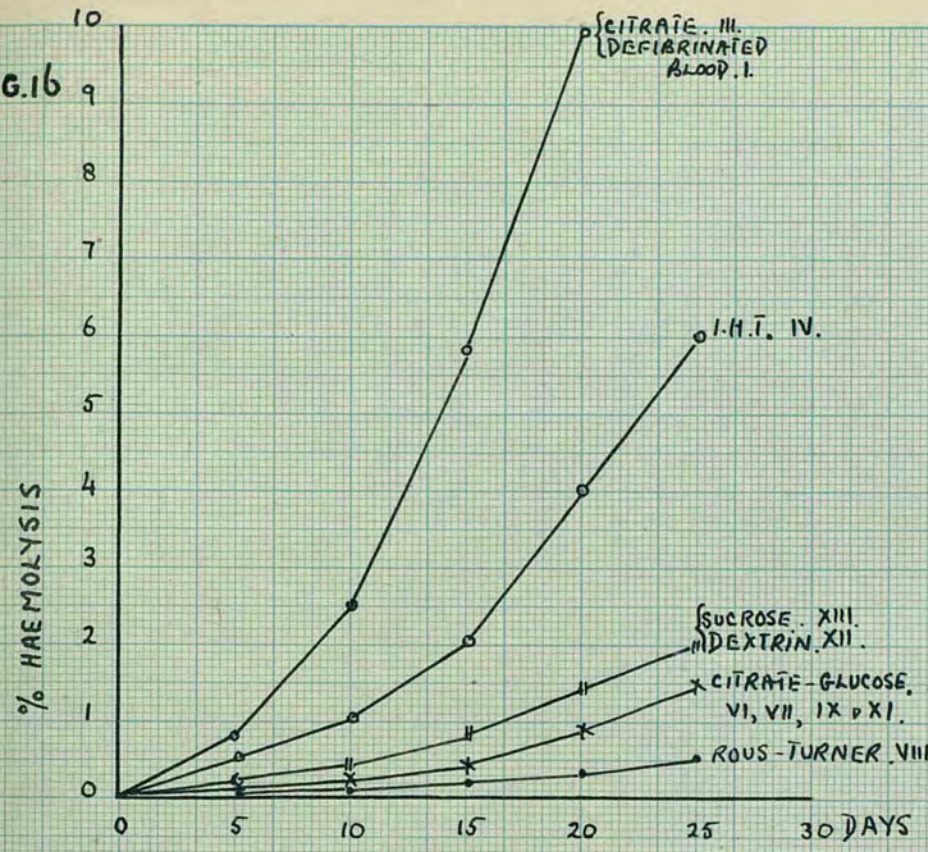
Spontaneous Haemolysis

Fig.16 confirms the results of those investigators discussed above. I.H.T. definitely inhibits haemolysis but its powers lag far behind those of sucrose, dextrin and glucose. Glucose is superior to sucrose and dextrin, a final concentration of 0.6% being just as efficient in delaying haemolysis as 2.4%. There is least haemolysis of all in the Rous-Turner mixture.

Osmotic Fragility

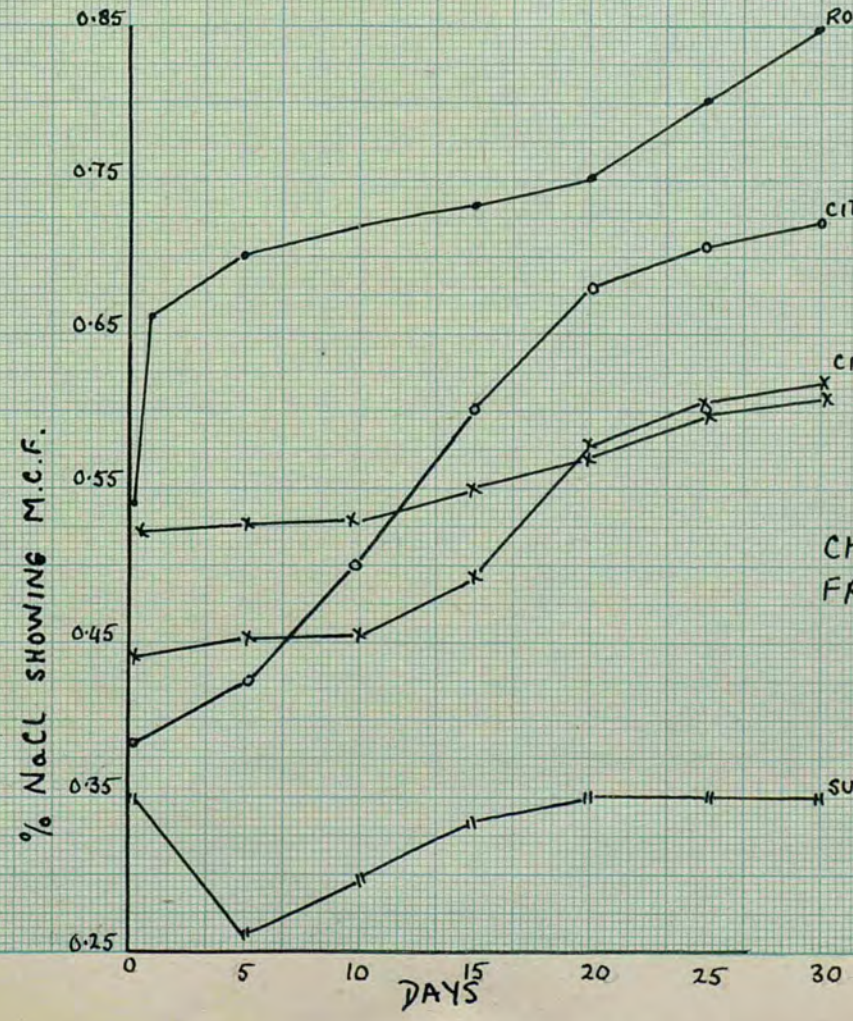
These results are again similar to those obtained by others. Glucose, in all concentrations tried, inhibited the gradual increase in osmotic fragility characteristic of cells stored without glucose. (Fig.17). It is most noticeable, however, that the initial median corpuscular fragilities of cells in

FIG. 16



CHANGES IN DEGREE OF SPONTANEOUS HAEMOLYSIS DURING STORAGE.

FIG. 17.



CHANGES IN OSMOTIC FRAGILITY DURING STORAGE

glucose solutions are higher than in the absence of glucose. The M.C.F. of cells in the Rous-Turner diluent may be, shown in 0.65% NaCl after only 6 hours storage. Sucrose causes a marked decrease in osmotic fragility which is succeeded by a gradual rise; the curve for dextrin is identical with that for 0.6% glucose.

It was noticed that the time at which the increase in osmotic fragility becomes more rapid, closely corresponds to the period when 'in vivo' survival starts to fall off. For instance, at 5 days for blood stored without glucose, 21 days for Rous-Turner blood and between 10 and 15 days for blood stored with 0.6% glucose, certain irreversible changes may set in causing degeneration of the red cell membrane.

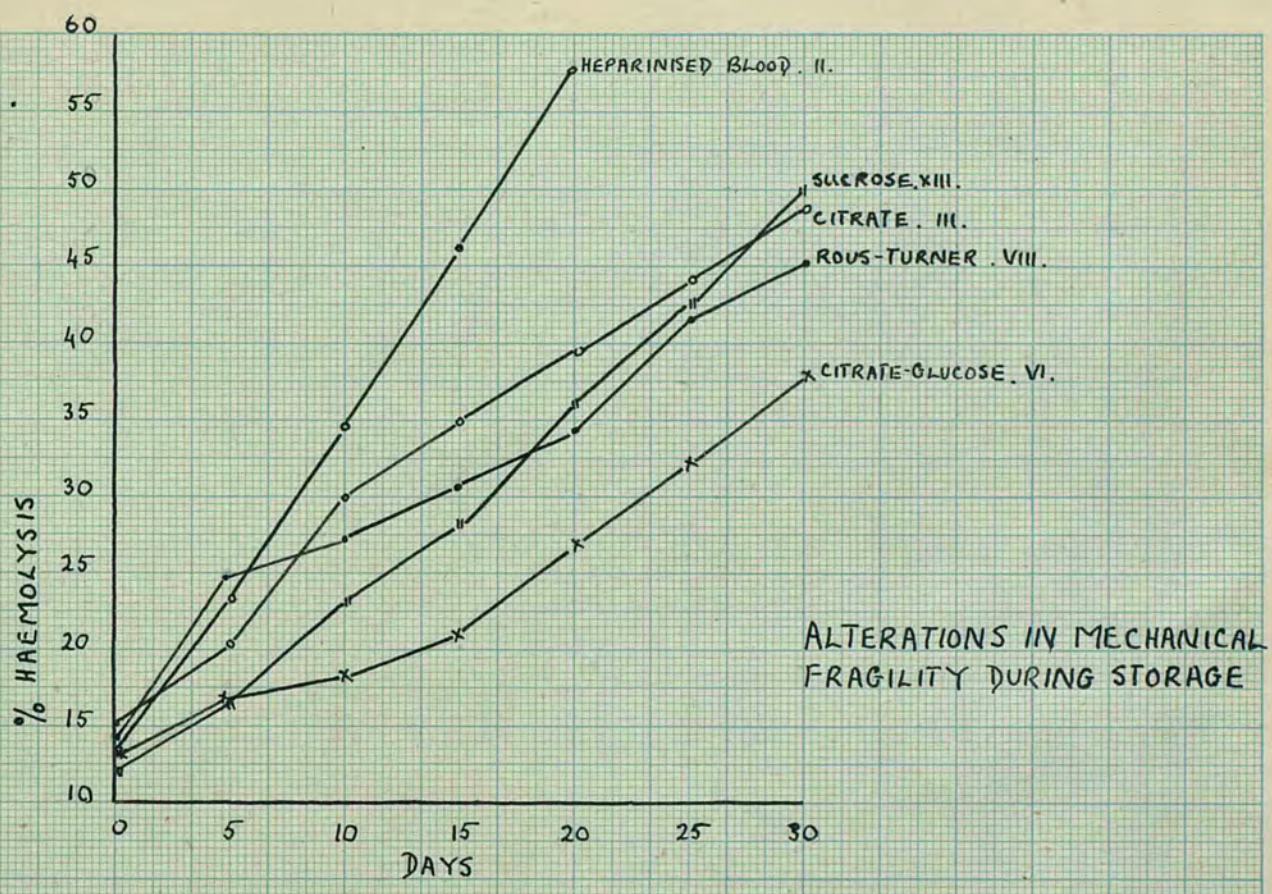
Mechanical Fragility

Again Rous and Turner first noticed that no constant relationship existed between osmotic ~~(1940)~~ and mechanical fragility. de Gowin (1940) also observed the increased resistance to mechanical shaking imparted by glucose, shown in Fig. 18 The raised mechanical fragility in Rous-Turner diluent may be associated with the very high osmotic fragility and swelling of the cells.

pH Changes

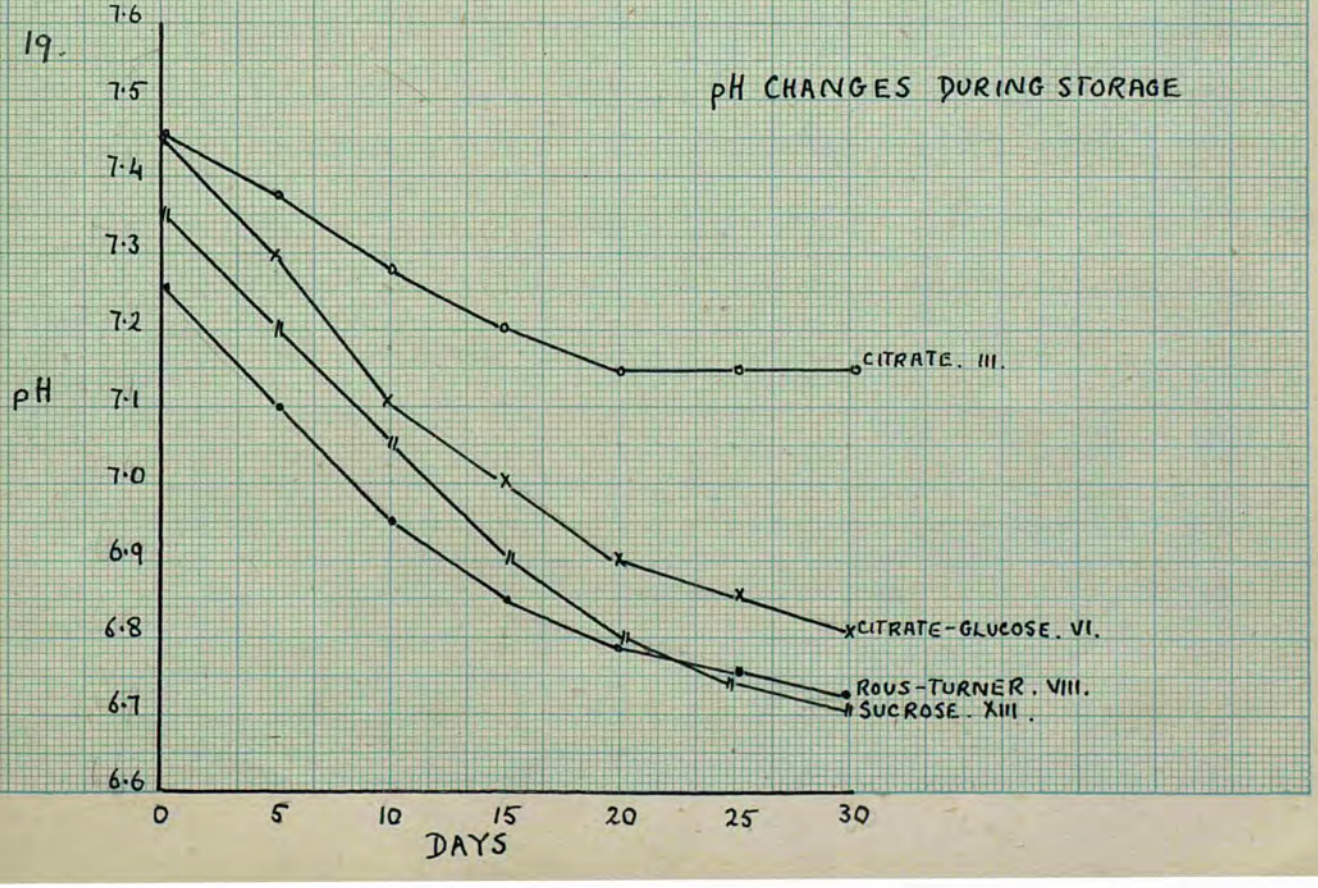
It was found that all the diluents were strongly alkaline (trisodium citrate was used); for instance the M.R.C. citrate glucose mixture had a pH of 9.3 but as Fig. 19 shows the

FIG. 18.



ALTERATIONS IN MECHANICAL FRAGILITY DURING STORAGE

FIG. 19.



pH CHANGES DURING STORAGE

added blood buffered this well, and the pH of the mixture falls to 7.45. Those bloods stored without glucose never showed a fall in pH below 7.0 during storage, while the pH of glucose containing bottles fell on an even slope, reaching pH 6.8 at the end of a month. The sucrose and Rous-Turner mixtures were slightly more acid.

Therefore such 'in vitro' tests as osmotic and mechanical fragility and change in pH can give no guide for good 'in vivo' survival; osmotic fragility tests especially may lead to completely fallacious conclusions. Certainly haemolysis is least in those diluents which give the best 'in vivo' survival and takes place more rapidly in worthless solutions. Haemolysis therefore may be a useful criterion in discarding these valueless diluents, but unfortunately sucrose and dextrin which are little better than citrate and I.H.T. for 'in vivo' survival inhibit haemolysis well, and so on this criterion, would not be discarded with the rest.

Fate of Fragile Cells

Fragile cells may be divided into two classes: those with increased osmotic fragility, such as the Rous-Turner cells, and those in which irreversible degenerative changes have started to take place. Haemoglobinaemia was encountered in two cases transfused with Rous-Turner blood and it seems very likely that the cells were so fragile that some haemolysed on

contact with fresh plasma. Intravascular haemolysis was never observed when blood that disappeared rapidly was transfused. That extravascular haemolysis had taken place was shown by the serum bilirubin rise. The extent of a rise in serum bilirubin depends upon the number of cells destroyed, the efficiency of the patient's liver and the time of blood sampling. Vaughan (1938) has shown that the maximum bilirubin rise occurs 5 hours after the transfusion. The results of the serum bilirubin changes (as estimated by van den Bergh's method), after many of the transfusions, are represented in table 4 . There was great variation in the individual results but whole groups show well that blood, stored without glucose, deteriorates much more rapidly than when it is present. This is a good qualitative confirmation of the 'in vivo' survival findings, and the table shows further the superiority of the Rous-Turner diluent.

It has long been known that potassium leaves the red cell during storage [(Kerr (1929), Ponder and Saslow (1931), Rowman, Oliver and Young (1940)]. Maizels and Whittaker (1940) noticed that the sodium contents of cells increased during storage, and that when these cells were transfused they caused an immediate rise in the level of cell sodium in the recipient. 24 hours afterwards the recipient's cell sodium

TABLE IV

Bilirubin changes in the recipient after Transfusion

Preservative	Time of storage in days	No. of cases	Serum Bilirubin mgm %			
			Before	0 - 2 hrs after	4 - 6 hrs after	24 hours after
A. Those containing no glucose.	5-10	19	0.1	0.5	1.2	0.3
	11-17	11	0.1	0.7	2.4	0.5
B. Citrate-glucose mixtures, except Rous-Turner.	0-5	15	0.0	0.3	0.5	0.0
	11-15	6	0.0	0.2	0.7	0.0
	17-21	16	0.1	0.7	1.3	
C. Rous-Turner	17-21	4	0.0	0.3	0.9	0.3
	24-28	4	0.1	0.3	1.1	0.3

TABLE V

Cell Sodium changes in the Recipient after Transfusion

Patient	Time of storage of blood given in days	Cell Sodium of blood given in mgm %	Cell Sodium, in mgm %, in Recipient's blood			
			Before Transfusion	After Transfusion	24 hrs after transfusion	48 hrs after transfusion
1	14	139	30.6	53	-	35
2	14	136	35	60	46	-
3	14	92	35	47	31	-
4	14	127	31	43	33	32
5	14	93	17	39	28	18

Sodium estimations were done on ashed samples of cells by the method of Sahlit.

had returned to its initial value through there was no parallel fall in the number of donor cells. Maizel's results were confirmed (Table 5), though unfortunately the survival of donor cells was not checked. Our present knowledge of cell survival can fill in the gap, for it is very unlikely that 14 day old blood would be completely destroyed during 24 hours in 5 cases. The removal of excess sodium and replacement by potassium must take place against a steep concentration gradient and cannot be explained as due to any simple physical process.

The fate of the 'fragile' Rous-Turner stored cells was first studied in the following way: a large transfusion of these cells was given to a patient, and at varying intervals after the transfusions the recipient's blood was agglutinated in the usual way. The free cells obtained, were separated from the agglutinates by filtration through a Whatman No.1 filter paper and sufficient collected to determine their osmotic fragility in one or two strengths of sodium chloride. This experiment proved to be invalid as is shown below.

The possibility that fresh plasma or saline could alter the properties of these cells 'in vitro' was next investigated. Some Rous-Turner blood was spun at a slow speed for a short time, and 1 cc. of cells, reasonably free from their own supernatant, were mixed with 2 cc. fresh heparinised plasma or 0.85% NaCl. From table 6 it is seen that within an hour the median corpuscular fragility is so decreased as to be within

TABLE VI

Changes in Median Corpuscular Fragility after reconstitution of Cells in Plasma or Saline

Diluent	Reconstitution in Heparinised Plasma			Reconstitution in 0.85% NaCl	
		% NaCl representing			
		M.C.F.	trace haemolysis	M.C.F.	trace haemolysis
Rous-Turner stored 21 days	On mixing	0.71	0.86	0.73	10% in 0.86%
M.C.F. 0.78% NaCl 20% haemolysis in 0.86% NaCl	1 hr after mixing	0.45	0.68	0.48	0.72
	21 hrs after mixing	0.45	0.62	0.49	0.68
Citrate Glucose X stored 19 days	om mixing	0.56	0.76	0.61	0.80
M.C.F. 0.65% NaCl trace haemolysis in 0.86% NaCl	1½ hrs after mixing	0.41	0.58	0.47	0.64
	4 hrs after mixing	0.39	0.56	0.45	0.60
Citrate glucose VI Stored 14 days M.C.F. 0.47% NaCl trace haemolysis in 0.60% NaCl	3½ hrs after mixing	0.43	0.56	-	-
Stored 21 days M.C.F. 0.50% NaCl trace haemolysis in 0.76% NaCl.	3½ hrs after mixing	0.42	0.64	-	-
Stored 32 days M.C.F. 0.68% NaCl trace haemolysis in 0.86% NaCl.	"	0.63	0.79	-	-
Sodium Citrate III stored 10 days M.C.F. 0.46% NaCl trace haemolysis in 0.76% NaCl.	"	0.42	0.67	-	-
Heparinised Blood Stored 13 days M.C.F. 0.68% NaCl trace haemolysis in 0.84% NaCl.	1½ hrs after mixing	0.67	0.80	-	-
Defibrinated Blood stored 12 days M.C.F. 0.46% NaCl trace haemolysis 0.78% NaCl.	"	0.46	0.74		

normal limits. This change has been noted by de Gowin (1940) and explains why many workers, using washed cells, have found no increased fragility of Rous-Turner cells.

Cells stored in other diluents were then tested in the same way to determine whether they too could not be 'reconstituted'. It was hoped that reversibility of fragility could be demonstrated in all cells which survived well 'in vivo' and that irreversibility would gradually become apparent as 'in vivo' survival fell off, perhaps, as mentioned before, at that point on the fragility curve where the slope steepens. It was found that cells with low osmotic fragility showed no change even after 24 hours. The osmotic fragility of cells from solutions containing glucose were always reversible, but as they increased in age, the capacity to reverse became less. It was difficult to obtain significant results with ~~young~~ blood stored for short periods without glucose, but certainly no reversibility was demonstrable in the specimens over ten days old.

Discussion

To Jacobs is due the idea that the resistance of cells to ~~hytenic~~ hypotonic saline haemolysis depends upon their power of swelling to accommodate a sufficient inflow of water so that the cell contents mey be rendered isoosmotic with the exterior. By taking up slack the biconcave disc becomes biconvex or

even globular and at a certain 'critical' volume loses its haemoglobin. Ponder and Saslow (1931) by direct measurement of cell radius, have shown that the cell first becomes spherical without increasing in its surface area, then gradually increases in size and haemolyses at a definite volume. Crosbie and Scarborough (1941) found that cells become spherical during storage and increase in volume; there are many who consider that this is the reason for haemolysis during storage.

The increased osmotic fragility, observed ultimately in any diluent during storage, may be due to one of four factors or a combination of any or all. Firstly hypertonicity of the diluent; ions and water may enter slowly (at 40°C) from the diluent in an endeavour to establish osmotic equilibrium, the cells would then gradually become more and more hypertonic to isotonic saline solutions and normal blood. Secondly, osmotically active substances, e.g. metabolites, may be liberated within the red cell from non-osmotically active substances, and these diffusing only slowly from the cell wouldncause swelling. Thirdly, the osmotic pressure of the haemoglobin inside the cell is many times greater than that of the diluted plasma proteins so that water may gradually pass into the cell because of this difference in colloidal osmotic pressure. Fourthly, degeneration of the red cell membrane will eventually take place. In most of the diluents there is at first a slow

rise in osmotic fragility followed by a more rapid rise, the onset of which, as has been mentioned before, occurs much sooner in blood stored without glucose. It is suggested that the first three factors operate slowly during the gradual rise, but when the cell membrane starts to deteriorate their influence becomes more pronounced. This was also suggested by Maizels (1940). The cells do increase in size during storage, their sodium content increases, and Maizels has shown that ester hydrolysis takes place within the cell, inorganic phosphorus rises and diffuses only slowly into the plasma.

The rapid increase in osmotic fragility of cells collected into the Rous-Turner diluent, and the smaller but significant rise found in all glucose solutions has quite a simple explanation. The question of the permeability of the red cell membrane to glucose has been very much disputed, but Klinghoffer (1940) and Maizels have shown that human erythrocytes are permeable. Glucose when added to blood in concentrations less than 1.5% is immediately evenly distributed between the water of cells and serum; at greater concentrations penetration is slower, but equilibrium is eventually reached. There cannot then be a solution of glucose isotonic with human erythrocytes. In the Rous-Turner diluent, increased fragility is due to dilution of the plasma diluent electrolytes by the

water of the so-called isotonic glucose, and as the water content of the cells increases so will the glucose content. When placed in normal saline or plasma these cells will lose water and glucose to their hypertonic surroundings, and the cell fragility will return towards its normal figure. It is extraordinary that cells can remain stretched for such long periods and yet maintain their biological function unimpaired.

Sucrose renders cells more resistant to saline haemolysis; ~~blood stored in this diluent~~ these cells sediments very rapidly ~~probably because the cells~~ and are ~~so~~ small, the mean corpuscular volume found is usually 65 μ^3. Since haemolysis does occur in this diluent it must be due to a factor other than bursting of the red cell membrane as a result of swelling.

The beneficial effect of glucose upon cell survival may be explained in several ways. (1) It may form a source of nutrition, thus maintaining cell metabolism and preventing degenerative changes from occurring in the cell membrane; in citrated or defibrinated blood the normal blood sugar level is reduced to 20-10 mgm% during 5 days storage and this level of reducing substances is then maintained. It may only be a coincidence that after five days storage deterioration in these diluents is rapid. When 1% glucose is added 0.5% still remains after 5 weeks storage. Maizels (1940) has shown that glucose inhibits the breakdown of phosphoric esters within the red cell, and thus the liberation of inorganic phosphorus.

It is possible that part of the metabolism of the cell is dependent upon the balance between phosphorylation and glycolysis, and when all the glucose is used up glycolysis ceases and resynthesis of phosphoric esters cannot take place.

(2) Blood stored with glucose becomes slightly acid. Maizels (1935) has shown that the permeability of red cells to cations is reduced in acid solution. He has also found that the degree of haemolysis is reduced in slightly acid solutions. Guest (1932) demonstrated that ester hydrolysis and synthesis within the red cells was very slow below pH 7.3. Perhaps then the general rate of metabolism and needs of the cell are reduced in weakly acid solutions.

(3) Maizels (1940) found that cells placed in glucose solutions had an increased extensibility, the maximum swelling in saline solutions, before haemolysis took place, being 64%, while with the addition of 1% glucose it rose to 84%.

It is not quite so easy to determine why the Rous-Turner diluent should be superior to the ordinary citrate glucose mixtures. The pH is slightly lower than in the other glucose mixtures so that the possible beneficial effects of lowered pH may be here enhanced. Although no routine sedimentation rates were performed it was noticed that the cells settled very slowly in these bottles. This decreased sedimentation rate is most probably due to dilution of the plasma globulin

and fibrinogen and the large volume of crystalloid present. [Fahraeus (1929).] Fahraeus (1939) showed that if red cells are allowed to sediment and then shaken up in their own supernatant plasma they possess an increased suspension stability; this he regards is due to the action of a substance which has been identified as lysolecithin. Lysolecithin is a very potent haemolysin which disperses the lipoprotein monolayer of the red cell membrane. Small quantities are formed in blood only when the cells and plasma separate into two layers. This work is unconfirmed, but it may be that lysolecithin is responsible for cell membrane deterioration during storage. To test this, a few of the M.R.C. citrate glucose bottles were kept well mixed during storage by carefully inverting them at suitable intervals so that sedimentation never occurred. The results were disappointing since no improvement in the 'in vivo' survival of the blood was found. However, there still remains the possibility that lysolecithin formation may be much slower in the Rous-Turner mixtures on account of the slower separation of the cells from the plasma diluent. Fahraeus also found that lecithinase responsible for the conversion of lecithin into lysolecithin is maximally active in weak alkaline solutions, the activity being very much reduced in acid solutions; the lower pH of all glucose mixtures would certainly be an advantage if red cell deterioration is due to the formation of lysolecithin.

The electrophoretic speed of red cells gradually increases as they mature and age and this is said to be due to increased absorption of ions on to their surfaces, and therefore impermeability to cations (Stephens 1940). Slow electrophoretic speeds are characteristic of red cells with slow sedimentation rates, red cells in glucose solutions (probably due to their decreased sedimentation rate) and red cells in acid media. Glucose and lowered pH may therefore help in the preservation of erythrocytes by maintaining their normal impermeability to cations.

The Rous-Turner diluent would be most impracticable for general use in blood banks (1) because it would have to be stored in 2 litre bottles; (2) the blood is so diluted that the supernatant plasma would always have to be removed before transfusion and (3) old blood could not be utilized as a source of plasma because the protein concentration is so low. Cells in diluent X however, containing 180 cc 5.4% glucose, 40 cc 5% sodium citrate and 320 cc of blood also sediments slowly and showed good 'in vivo' survival at the critical period of 18-20 days storage (see Table III).

Summary

(1) A method for following quantitatively the survival of transfused cells in a recipient is described. It is applicable to the determination of the fate of stored

blood, and the fate of blood in many pathological conditions. It may also be used in the estimation of blood volume.

(2) Fresh erythrocytes transfused may be recognized in the recipient during 100 days.

(3) Blood stored up to 21 days in the M.R.C. citrate glucose mixture loses little of its therapeutic value. Thereafter deterioration is rapid. Deterioration of cells occurs after 5 days storage without glucose.

(4) Blood stored in many diluents, recommended on 'in vitro' criteria, were tested for percentage haemolysis, osmotic and mechanical fragility and pH changes, to determine whether any correlation existed between 'in vivo' and 'in vitro' properties. Bloods with a high percentage of haemolysis may be discarded. Osmotic fragility tests may be a very fallacious guide to 'in vivo' survival. It was found however that the high osmotic fragility, shown by cells stored in some glucose solutions, was reversed by plasma or isotonic saline. Changes in mechanical fragility were no guide to 'in vivo' survival.

(5) The beneficial effects of glucose on 'in vivo' survival are associated with the maintenance of normal cell metabolism, decrease in the pH of the mixtures and increased extensibility of the red cell membrane.

(6) Rapid deterioration of blood in the Rous-Turner diluent does not begin until after one month's storage. The superiority of this diluent may be associated with the slow sedimentation rate of the cells and therefore the slower formation of lysolecithin.

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Sutton 1

PARTITION OF POTASSIUM IN STORED BLOOD

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The increasing use of stored blood for transfusion purposes calls for an extension of our knowledge of the stability of the human red cell when kept for long periods *in vitro*. In general the normal properties of the red blood corpuscle appear to be best preserved in undiluted human plasma ; but an indication of rapid changes in the chemistry of shed blood is found in work upon blood potassium, immediate separation of the formed elements being necessary if a true estimate of the plasma potassium is to be obtained (Myers and Short, 1921 ; Wilkins and Kramer, 1923 ; Dulière, 1931a, 1931b). Recently Scudder, Drew, Corcoran, and Bull (1939) found a rise of plasma potassium when human blood was kept at 2°–4° C. The reported increase was so large that it seemed necessary to repeat the work and endeavour to find a practical method of limiting these changes. A solution to this problem is likely to prove difficult, since Scudder *et al.* also showed that the change in plasma potassium content was independent of the type of anticoagulant used, and that it took place in blood stored, without admixture, under oil.

We here record variations of plasma potassium level in blood stored according to the current practice of the Medical Research Council's Blood Supply Depots. The relation of these changes to haemolysis and the influence of certain physical factors are also discussed.

Plasma Potassium Level

360 c.cm. of blood from the antecubital vein was collected in the standard M.R.C. bottle, coagulation being

prevented by the addition of 180 c.cm. of 1.05 per cent. sodium citrate in 0.85 per cent. sodium chloride. The bottles were capped and stored at 2°–4° C. in the dark (Medical Research Council, 1939; Vaughan, 1939). Sampling, with aseptic precautions, was preceded by thorough mixing of the blood by repeated inversion of the bottle. Formed elements were removed by centrifuging for ten minutes at 4,000 r.p.m. and respinning the supernatant plasma. Control experiments, in which further spinning was carried out, indicated the removal of all red cells. On completion of a series of estimations on any sample of blood the blood remaining in the bottle was tested for infection by incubation at 38° C. for forty-eight hours, with subsequent subculture to broth or agar slopes.

Potassium was estimated by the method of Kramer and Tisdall (1921a). A preliminary series of estimations showed that traces of free haemoglobin caused considerable error, the apparent potassium sometimes far exceeding the whole-blood figure, a fact which had been noticed also by Dulière (1931a). All subsequent estimations, therefore, were made on two aliquots of a trichloracetic acid filtrate, Kramer and Tisdall (1921b) having shown that the presence of this acid does not affect the accuracy of the method. The figures quoted throughout this paper represent the concentration of potassium in the plasma-anticoagulant mixture.

Results.—(1) Eighteen bottles of blood were sampled daily for fourteen days. In each the plasma potassium increased rapidly in the first week, averaging 102 $\pm$ 17 mg. per 100 c.cm. at seven days. The process then slowed up, and throughout the second week the values gradually approximated to the whole-blood figures (Fig. 1). It was difficult to prevent infection of the blood by air-borne organisms when bottles were opened frequently. However, it was found that there was no difference of rate of potassium repartition between infected and non-infected samples.

(2) Random samplings of bottles kept in stock also showed that the plasma potassium concentration is related to the time of storage. The scatter of values indicated a rapid rise in the first week, up to 80 $\pm$ 16 mg. per 100 c.cm. at seven days. As before, subsequent estimations showed that the figures approximate to the whole-blood level, though uniform distribution of potassium between cell and plasma may not be obtained even after three months (Fig. 2). The mean potassium value in this

series was lower than when the bottles were opened daily ; this may be associated with the trauma caused by the repeated disturbance of bottles in the first series.

Conclusions.—The distribution of potassium in blood stored in the manner already described changes progressively from the time of shedding. The shift of the

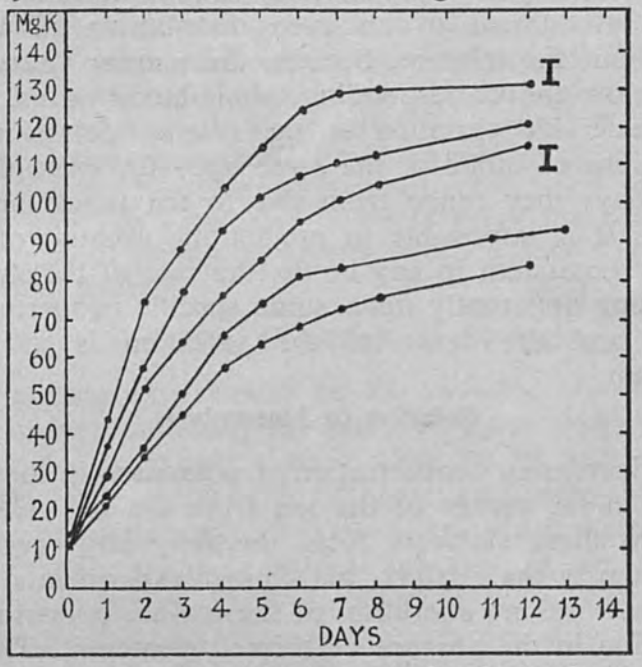

FIG. 1.—Plasma potassium concentration in bottles sampled daily. Ordinate = mg. potassium per 100 c.cm. Abscissa = time of storage in days. Temperature 2°–4° C. " I " indicates bottles infected with air-borne organisms.

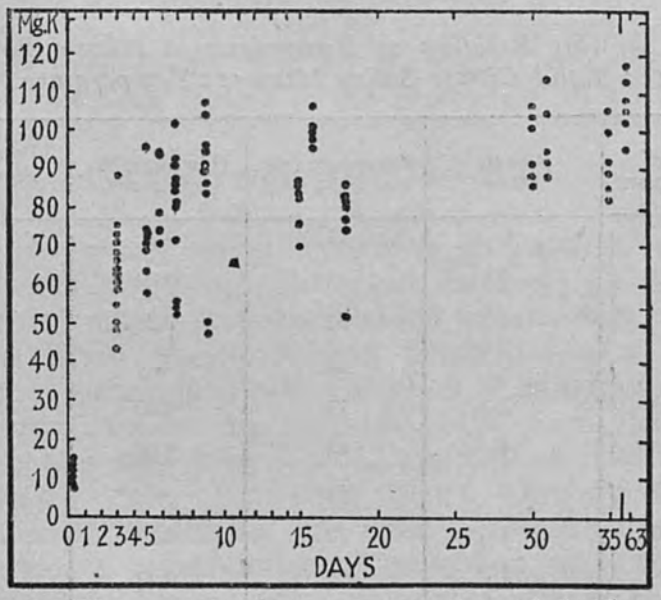

FIG. 2.—Plasma potassium concentration of bottles taken from stock at random. Ordinate = mg. potassium per 100 c.cm. Abscissa = time of storage in days. Temperature 2°–4° C.

cation is very rapid during the first few hours: initially 10 mg. per 100 c.cm., the concentration in the plasma may rise to 15–20 mg. per 100 c.cm. within thirty minutes. The graphs show two phases of redistribution, the rate being relatively rapid throughout the first week and slower after seven days. The flattening of the curves is not due to the attainment of an even distribution of the ion throughout the mixture, because the plasma value is still well below the corresponding whole-blood value. There is considerable variation in the plasma potassium concentrations of blood of the same age—for example, after seven days they range from five to ten times the initial figure. It is impossible to predict the amount of extracellular potassium in any bottle, the rate of redistribution depending apparently upon some specific property of the blood, and the cause of the variation is at present unknown.

Relation to Haemolysis

The increasing concentration of potassium in the plasma is due to the escape of the ion from the red cells. Not only do these elements form the only large reserve of potassium in the mixture, but control experiments showed that there was no alteration of the plasma potassium concentration in the absence of formed elements. The basic mechanism may involve (*a*) haemolysis, the cell losing both haemoglobin and salts, or (*b*) movement of the cation alone through an altered membrane. The follow-

Table showing Relation of Haemolysis to Plasma Potassium Increase : Blood–Citrate–Saline Mixture ; Temperature 2°–4° C.

No. of Experiment	Age in Days	Potassium (mg. per 100 c.cm.)	Haemoglobin gramme %	Potassium Equivalent to Free Hb
678	0	11	—	—
	7	78	0.04	0.6
	14	92	0.20	2.8
679	0	13	—	—
	7	73	0.03	0.4
	14	88	0.08	1.0
680	0	11	—	—
	7	72	0.07	1.0
	14	90	0.17	2.0

Right-hand column shows theoretical increase of plasma potassium (in mg. per 100 c.cm.) equivalent to estimated loss of haemoglobin from red cells.

ing observations prove that the plasma potassium concentration is not an index of haemolysis:

1. Potassium and haemoglobin concentrations in the plasma vary independently: a more deeply stained plasma may actually contain less potassium than one which is free of pigment.

2. The potassium may increase up to seven times the initial level before haemolysis can be detected by the naked eye.

3. Direct estimations of the haemoglobin in plasma were made by matching against known dilutions of laked blood. The observed haemolysis accounted for a small proportion of the extra potassium only. For example, in Experiment 678 (see table) the liberation by red cells of 0.2 gramme haemoglobin into 100 c.cm. plasma would entail the simultaneous appearance of 2.8 mg. potassium, which is only 3 per cent. of the actual figure.

Altered Conditions of Storage

In all the experiments so far reported the blood was kept under conditions far removed from those obtaining in the body. It was venous when taken, and could not undergo any appreciable gaseous exchanges before the bottle was sealed off. It was cooled to a low temperature and diluted with a watery solution of foreign salts. Previous workers have shown that these alterations of environment cause a change of permeability of the cell membrane. Jacobs and Parpart (1931) investigated the effect of oxygen tension and temperature changes on the osmotic resistance of the erythrocyte, and Kerr (1929) followed the movement of potassium between cell and plasma upon the addition of various diluents. The following experiments were an attempt to estimate the part played by such factors in the production of the observed changes.

Dilution and Salt Concentration.—Ten samples of blood were studied as follows: (1) three were received on to enough sodium citrate crystals to give a final concentration of 0.305 gramme/100 c.cm. mixture; (2) three were collected into 3 per cent. sodium citrate, 40.8 c.cm./360 c.cm. blood, giving a final concentration as before; (3) four were mixed with a solution of heparin (liquemin), 2.5 c.cm. (= 10 mg. heparin) /150 c.cm. blood. All the bottles were kept at 4° C. and plasma potassium was estimated every forty-eight hours. Reduction of the volume of diluent did not cause significant alteration of the rate of redistribution of potassium, since the figures obtained in Experiment 2 showed a rise in the plasma to 129–132 mg. per 100 c.cm. in seven days. A similar result was obtained in Experiment 1, where sodium citrate

crystals were used as the anticoagulant. The use of heparin in Experiment 3 likewise failed to affect the usual rise in plasma potassium, although the plasma dilution was negligible and there had been no addition of foreign salts (Fig. 3).

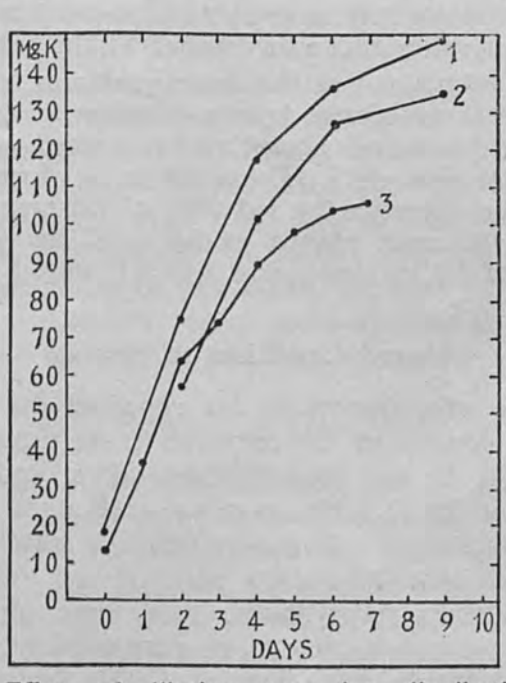

FIG. 3.—Effect of dilution upon ion distribution. Anticoagulant 1 = solid citrate ; 2 = 3 per cent. citrate (initial estimate unreliable); 3 = heparin. Ordinate = mg. potassium per 100 c.cm. Abscissa = time in days. Temperature 2°–4° C.

Oxygen Tension.—Four samples of blood were taken into the usual citrate–saline solution. The air in the bottles was replaced by oxygen at two atmospheres pressure, the blood rapidly becoming bright red with repeated inversion of the bottle. The samples were kept at 2°–4° C., the oxygen pressure being maintained. The plasma potassium showed the usual rapid rise to a high level, averaging 82 mg. per 100 c.cm. after six days (Fig. 4). Saturation with O_2 under these conditions does not reduce the loss of the ion from the red cell.

Temperature.—Eight samples of blood were taken into citrate–saline and studied as follows: (1) two were kept at 38° C. ; (2) six were kept at room temperature, protected from draughts. Each bottle in both series was sampled every forty-eight hours. At 38° C. the plasma potassium concentration rose rapidly to 140–150 mg. per 100 c.cm. in six days. On the other hand, in the samples kept at room temperature the rise was significantly less

rapid than in any kept at 2°–4° C. The graph for the first week of storage is practically a straight line, the levels reaching only 44 ± 7 mg. per 100 c.cm. in six days (Fig. 4).

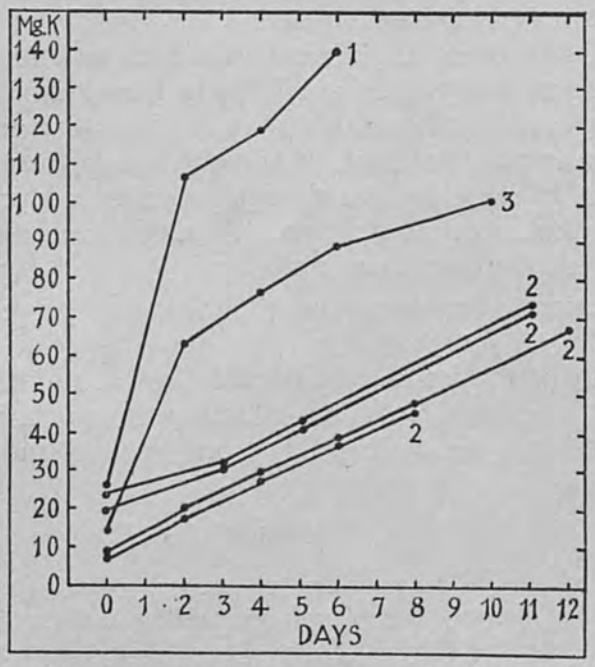

FIG. 4.—Variation of temperature and of oxygen tension. 1 = bottle stored at 38° C.; 2, 2 = bottles stored at room temperature; 3 = storage under two atmospheres oxygen. Ordinate = mg. potassium per 100 c.cm. Abscissa = time in days.

The bottles were sampled by a closed method, and air entering was passed through a Jena glass filter. In spite of these precautions all the bottles of this series were found to be infected with air-borne organisms to an extent which prevented accurate assessment of the influence of temperature on the repartition of potassium. The findings do suggest, however, one means of slowing up the process, although obviously this would only prove useful if infection of the blood could be prevented absolutely.

Discussion

Our results confirm those of Scudder *et al.*, and show that the total amount of extracellular potassium in M.R.C. bottles stored at 2°–4° C. may be 0.2–0.4 gramme within one week. This change is much greater than any other yet demonstrated in blood during the first two weeks of storage; and, whatever the clinical interest, it does provide one useful index for comparing different methods of preserving blood for transfusion purposes.

It is known that potassium salts are toxic when administered in large amounts, the ion affecting particularly the cardiovascular system—for example, Thomson (1939) has recorded pronounced changes in the electrocardiogram when potassium salts were administered per os. There is, however, no clinical evidence that the amount of potassium likely to be available in the volume of blood generally used for transfusion would be sufficient to produce toxic manifestations. Indeed, the absence of toxic symptoms in cases receiving large volumes of stored blood suggests that this repartition of potassium may be of minor clinical importance only.

The desire to find a means of limiting or preventing the repartition of potassium is only partially realized. The movement of the ion is considerably less at room temperature, but the possibility of infection is much increased and introduces an undesirable complication unjustifiable in practice.

Summary

In blood stored according to current Medical Research Council specifications the plasma potassium concentration rises rapidly during the first week to five to ten times the initial level ; thereafter the rise is comparatively slow.

The source of this potassium is the red cell, from which it is liberated quite independently of haemolysis.

At room temperature the plasma potassium concentration rises much less rapidly, reaching only 44 ± 7 mg. per 100 c.cm. in six days.

Reduction of the volume of diluent, absence of foreign salts, increase of oxygen tension, or storage at 38° C. did not reduce the rate of redistribution of the ion.

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By PATRICK L. MOLLISON and I. MAUREEN YOUNG. From the S.W. London Blood Supply Depot.

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ON THE SURVIVAL OF THE TRANSFUSED ERYTHROCYTES OF STORED BLOOD.

By Patrick L. Mollison and I. Maureen Young. From the S.W. London Blood Supply Depot.

(*Received for publication 30th September* 1940.)

The opinion that the erythrocytes of stored blood do not survive long after transfusion has been expressed by many authors; thus Riddell [1939] considers that because of the increased fragility of the cells of blood that has been stored for more than four days, "it is very doubtful whether it would exert any prolonged therapeutic effect." Marriott and Kekwick [1940] sum up available evidence as follows: "Red cells of blood conserved for more than a week by present methods do not last long in the recipient." Again, Edwards and Davie [1940] state: "Aged erythrocytes and those in which the fragility has been increased have only a short effective span of life and can therefore have little lasting value."

These statements are, perhaps, rather surprising, since in 1916 Peyton, Rous, and Turner [1916], from their experiments on rabbits, concluded that cells stored up to two weeks function normally after transfusion. This conclusion was reached after studying blood counts and estimating the excretion of hæmoglobin and bile pigments, etc.

In March of this year we had the opportunity of examining some results obtained by Dr. J. O. Oliver, then Director of this Depot, and Dr. G. L. Taylor of the Galton Laboratory Serum Unit. They followed seven cases by the direct differential method (referred to below), using M and N agglutinating sera. They were able to show that transfused erythrocytes of stored blood survived for some weeks after transfusion. In one patient, for instance, who received 11-day-old blood the donor cells could be identified by direct agglutination after 51 days.

The object of this investigation has been to extend these observations further and in greater detail and to apply to the problem a method capable of giving quantitative results. Methods of following quantitatively the fate of transfused erythrocytes are all based upon the method described by Ashby [1919 *a*]. This, in brief, consists of the transfusion of cells of Group O to a recipient of Group A. After transfusion the recipient's blood contains two types of cells, O and A. The A cells can be "removed" with alpha serum and the free (O) cells

counted. Actually a small fraction of the A cells are not agglutinated, and constitute a blank value to be deducted from the total of free cells.

Wiener [1934] has adopted the method using the sub-groups M and N. For example, if cells of Group M are transfused to a recipient of Group N, the N cells can be agglutinated and the free (M) cells of the donor counted. Considerable criticism has been levelled both at the general validity of these methods and also at the reliability of the technique. Isaacs [1924] suggested that following transfusion a number of immature cells would be thrown into the circulation, and being, as he thought, inagglutinable, would simulate donor cells. Ashby [1924], however, showed that the extent of the rise in the inagglutinable count depended upon the quantity of blood transfused and upon the patient's body-weight. Furthermore, this rise did not occur when a patient of Group A received a transfusion of Group A blood but only when Group O blood was transfused.

In addition, if blood of Group M is transfused to a patient of Group N a further refutation of Isaacs' theory may be made. Before the transfusion, agglutination with anti-N serum will leave free only a small fraction of the patient's cells—the blank "inagglutinable count." Following the transfusion a greatly increased number of free cells will be found. If now a sample of the recipient's blood is agglutinated with anti-M serum in addition, it will be found that this excess is "removed" and only a small fraction of free cells, equivalent to the initial blank figure, is left. We have performed this experiment several times and always with the same results.

The reliability of the technique has been carefully considered by Jervell [1924], who reached the conclusion that the method of Ashby could yield important quantitative results. He considered that the error of the method was plus or minus 10 per cent., but was careful to point out that the potency of the sera used and the effects of temperature, concentration, etc., are of great importance. A convincing demonstration of the quantitative nature of the results may be made by performing agglutination of the same cells with two different sera (viz. of the ABO and MN systems). A description of such an experiment is given in the Appendix.

The object of the work here described has been primarily to perform quantitative estimations of the numbers of donor erythrocytes surviving at various periods after the transfusion of stored blood. In addition an attempt has been made to support the contention that the figures have a quantitative significance.

The Blood used for Transfusion.

In every case blood was taken from supposedly healthy donors into a diluent consisting of 1·05 per cent. sodium citrate in 0·85 per cent.

saline. 360 c.c. of blood were taken into 180 c.c. of this diluent. Sufficient glucose was added to the citrate saline mixture after autoclaving to provide a final concentration of 1 per cent. in the blood mixture.

Since many transfusions were of the O to A type, a suspension of cells only was used to avoid the possible transfusion of a large bulk of high titre agglutinins. This can be very conveniently accomplished since stored blood sediments quite rapidly and the supernatant plasma may be removed. In most cases the cells were resuspended in 1·1 per cent. saline with 1 per cent. glucose as this solution produces minimal hæmolysis [MacQuaide and Mollison, 1940]. In a few cases the cells were given in a minimal quantity of plasma. Different quantities of this cell suspension were given to different patients, but usually the equivalent of two bottles of stored blood (*i.e.* 720 c.c. of whole blood) was given. The blood was stored at 4° C. for various periods (1–29 days) before transfusion. The blood was usually given at approximately 3–10 c.c. per minute.

Clinical Material.

The majority of the patients transfused were suffering from anæmia connected with pregnancy or parturition. A certain number were delivered whilst they were still under observation, and an indication of this is given in the figures.

Methods.

The experiments can be divided into three groups:

1. Simple O to A or M to N transfusions.

The results of these experiments have been combined in figs. 1 and 2.

2. A combination of the above two methods in the same patient. For example, patient A. C. belonged to Group BN, and was transfused with blood of Group OM. The donor erythrocytes could be counted after agglutination of the recipient's cells either with beta serum or with anti-N serum, neither of these sera agglutinating the donor erythrocytes. The parallelism between the two sets of figures is striking. We consider that the three cases followed in this way afford strong evidence of the quantitative validity of our other results. Martinet [1938] used a similar method as a check upon his experiments, using the M and N sub-groups. However, his experiments were only qualitative.

As mentioned above, we also applied this method to our *in vitro* experiments, and an example is given in the Appendix.

3. The measurement of the survival of two different transfusions in the same patient:

(*a*) Transfusions of two different "ages" of blood.
(*b*) Transfusions of blood stored in two different ways.

These experiments are in the nature of controls.

The simplest method illustrated by the patient E. (see fig. 4) is to give a second transfusion at a sufficient interval after the first and when all the cells of the first transfusion have been destroyed.

This method involves observing a patient for many months, and the patient's ability to deal with the transfused cells may have altered in the meantime. To avoid these difficulties, if the recipient is of Group AB two transfusions may be given simultaneously and followed separately. This can be most simply done by giving one transfusion of Group A and one of Group B. It may also be done by giving some blood of Group O and some of Group A; thus, in the patient D. S., a transfusion of 4-day-old blood of Group O was followed by a transfusion of 21-day-old Group A. After these two transfusions agglutination with beta serum left free the cells (A plus O) of both transfusions. With alpha serum only the O cells of the first transfusion was left free. By simple arithmetic the number of A cells surviving could be counted.

Since persons of Group AB do not amount to more than 3·2 per cent. of the London population [Wiener, 1939], a more generally applicable method is the use of the M and N sub-groups in addition. Thus, if a patient of Group AM is transfused (i) with OM blood, and (ii) with AN blood, agglutination with alpha serum will leave free the OM cells of transfusion (1), and agglutination with anti-M sera will leave free the AN cells of transfusion (2).[1] The two transfusions can be given immediately one after the other (a little saline being washed through the apparatus in between).

Although we have performed all our experiments quantitatively, we have in some cases used the method of "Direct differentiation" [Dekkers, 1939] to determine the "end-point." This method consists in the identification of the donor cells by direct agglutination, using the M and N sub-groups. Thus, when the number of inagglutinable cells falls to a figure which is but little higher than the original blank value, a decision as to whether or no donor cells are still surviving can be made by testing for the presence of the foreign agglutinogen. Such a combination of methods was first suggested (but not applied) by Martinet [1938].

As an example, an asterisk has been inserted in fig. 6. At this point, 84 days after the transfusion only 45,000 cells (per bottle) could be attributed to the donor by the quantitative method, and one cannot be certain that this number exceeds the possible variations in inagglutinable count. A test for the agglutinogen M was therefore made, the recipient and the donor of the second transfusion both belonging to Group N. The finding of small agglutinates showed that the cells of the first transfusion were still surviving.

Technique.

Since we did not adhere to the details of the technique originally described by Ashby, we feel that it is necessary to give our reasons for the changes which we adopted.

Briefly, Ashby's technique consists in taking blood into a leucocyte

[1] Since the completion of this work, we have learned that Wiener and Peters [1940] used this identical method to compare the survival of two transfusions in the same patient, though for a different purpose.

pipette and then drawing up agglutinating serum so that a final concentration of blood/serum of 1/22 is obtained. The mixture is expelled into a tube and incubated (with shaking at intervals) for 40 minutes. The tube is placed in an ice-box overnight and then counts of the free cells in the fluid are performed.

Our criticisms of this technique fall under four headings:

(1) The concentration used. A concentration of 1/22 makes blood-counting very difficult when more than 600,000 cells per cu. mm. are present. Furthermore, Rouleaux formation is definitely commoner at this concentration and further interferes with counting.

(2) The temperature suggested for incubation is 37° C. This is difficult to understand, since it is well known that warming hinders agglutination. Jervell, too, was puzzled by Ashby's choice of temperature, and actually performed his agglutinations in a refrigerator. This, however, is open to the disadvantage that it provides an opportunity for the action of cold agglutinins (as noted later by Ashby, 1919 *b*). We therefore performed our experiments at room temperature.

(3) Forty minutes does not seem to be an adequate time for agglutination. Taylor [1938] suggests that two hours is the optimum.

(4) It is well known that flat-bottomed vessels favour agglutination, and therefore the tubes recommended by Ashby were replaced by small bottles of such a size that the amount of fluid (0·2 c.c.) formed a thin film upon their floor.

With these considerations in view we adopted the following technique: 20 cu. mm. of the blood to be tested was taken from the patient in a hæmoglobin pipette and blown into 1 c.c. of citrate saline. From this suspension (1/50) 0·1 c.c. was taken and mixed with 0·1 c.c. of the appropriate serum in a small flat-bottomed bottle (as used for performing a red cell count). (0·1 c.c. only was used simply because large quantities of reliable anti-M and N sera are not available.)

The mixture was left for 2 hours at room temperature with brief shaking at half-time. At the end of 2 hours the bottle was shaken vigorously and a drop of the fluid withdrawn and placed in a Burker-counting chamber. This chamber was chosen because of the large area of the ruling. This enabled a selection of columns to be made, when large agglutinates obscured part of the field.

All the experiments were performed in duplicate, and counts in every case were made by both of us simultaneously.

To determine what constitutes an "agglutinate" is not easy, particularly when pairs are seen. We found it helpful to examine control experiments in which no agglutinating serum was present. This enabled the pairs which were stuck together to be distinguished from those that

are agglutinated. Discoloration of cells is always held to indicate agglutination. When the difference in parallel experiments was greater than expected the counts were repeated, and the pair showing the best agreement were selected. When any gross discrepancy was found agglutination was performed again.

Görl [1926] has objected that shaking may increase the number of free cells considerably. This is not supported by a theoretical consideration of the subject, for the attractive force between agglutinated cells is very great. Moreover, we have found that moderate shaking for fifteen seconds or less does not significantly raise the number of free cells. Furthermore, we have frequently found very few inagglutinable cells after vigorous shaking. Jervell advises shaking for one minute, and found no significant rise even after two minutes' shaking.

The titre of the serum was found to be of the greatest importance in securing consistent results. We were fortunate in obtaining sera of very great potency from Dr. G. L. Taylor of the Galton Laboratory Serum Unit. The alpha serum used throughout had, for example, a titre of 1/256, and the beta of 1/512.

We have found that cells of the Sub-group A_2 are difficult to agglutinate completely with ordinary grouping sera. Even with sera of high titre the "inagglutinable count" of A_2 cells is usually higher than that of Group A_1. Therefore A_2 cells provide a useful control in the selection of a suitable alpha serum. An alpha serum that will give an inagglutinable count of less than 60,000 cells per cu. mm. with Group A_2 blood may be considered satisfactory. Higher figures previously recorded for the blank "inagglutinable count" are mainly, we suggest, due to working with sera of insufficient potency.

The M and N agglutinating sera are not, on the whole, so reliable as those of the ABO system—presumably because they are "absorbed" sera. Thus some samples prove to be of insufficient potency to produce complete agglutination and others give non-specific reactions. The latter can usually be avoided by dilution with an equal volume of saline, and we adopted this precaution throughout and also worked with controls. Some patients were followed with three different sera, but eventually we worked only with one reliable anti-M and one anti-N, and the experiments in section (3) show the good quantitative agreement with the results obtained with alpha and beta sera.

Results.

We have expressed our results graphically, plotting the number of cells surviving against the time since the transfusion. In every case we performed agglutination on a sample of the patient's blood before transfusion, and subtracted this "blank" value obtained from the number of free cells found after transfusion. In the figures which

follow, this net total was then divided by the number of bottles of stored blood given to the patient.

The initial figure after transfusion depends upon many factors:

(1) The donor's red cell count and the volume of blood transfused.
(2) The recipient's blood-volume.
(3) The amount of destruction of donor cells that has already occurred before the sample is taken.

The first factor was partially standardised, as mentioned above, by dividing the number of surviving cells by the number of bottles of blood given.

We hoped to minimise blood-volume changes by using concentrated suspensions of red cells. Although the commencing destruction of

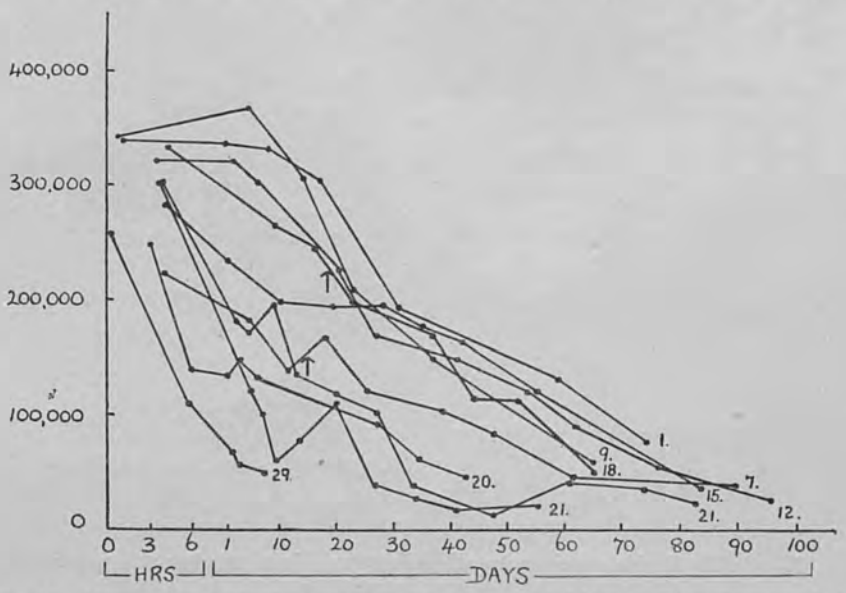

FIG. 1.—Survival of single transfusions of stored blood. The figures at the end of the curves indicate the length of storage in days. The initial figure depends upon many factors (discussed in the text), but after taking these into account, the slope is the important index of survival.
An arrow indicates that at this point the patient was delivered of a child.

effete red cells introduces a certain error, yet if the first sample is obtained within three to four hours of the mid-point of the transfusion, the expected number (approximately) is found except with blood more than three weeks old. (Example below.)

From fig. 1 it will be seen that, on the whole, the figures fall upon an even slope, and further that this slope is much the same with all the blood stored for less than 18 days. However, with the older blood the destruction is more rapid.

In two cases in which blood of 20 and 29 days old respectively was given, different samples were taken within the 6 hours following transfusion, and it was found that that period is a time of rapid destruction.

The fact that the transfusion of stored blood is followed by an immediate rise in bilirubin agrees well with these observations. For instance, in the patient who received 20-day-old blood (see fig. 1), 3 hours after the transfusion 594,000 cells per cu. mm. attributable to the donor were found and the bilirubin had already risen from 0·8 to 3·0 mg. per cent. Six hours after the transfusion only 274,000 cells per cu. mm. remained, and the bilirubin had risen to 4·5 mg. per cent. Six days later, however, there were still 250,000 donor cells per cu. mm. It should be pointed out that this is a considerably greater initial destruction than occurred in the majority of the cases.

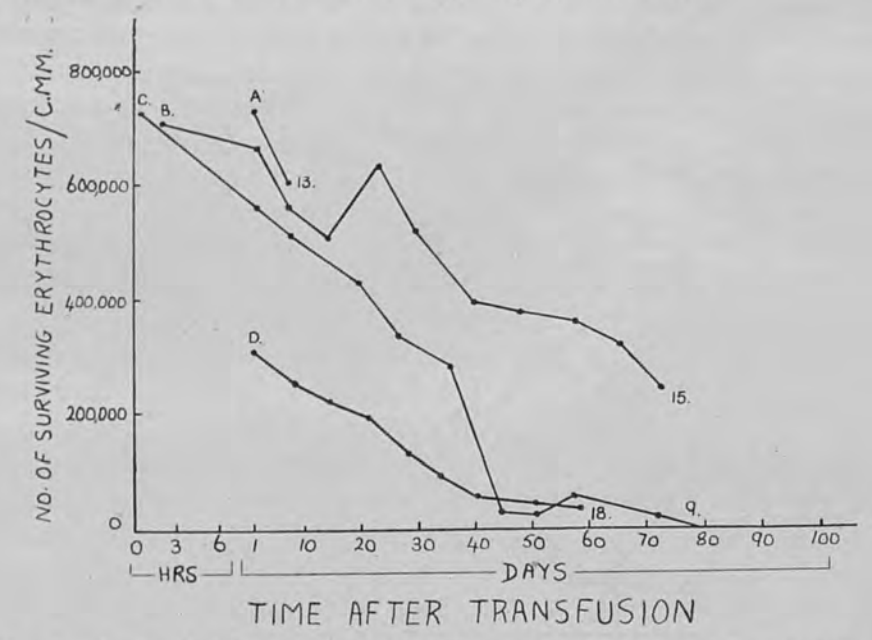

Fig. 2.—As fig. 1, but recipients all children. Note greater rise per bottle of stored blood.
Weight of children: A., 3 stone; B., 3·5 stone; C., 4·0 stone; D., 4·5 stone.

The total life of the transfused erythrocytes of blood stored for less than 18 days appears to be at least 70 to 90 days.

Fig. 2 shows that much greater rises in the number of donor cells per cu. mm. are produced by equivalent amounts of blood when given to children. Furthermore, the rise produced is approximately inversely proportional to the body-weight. The life in two of these four cases seems to be a little shorter, viz. practically all donor cells have disappeared at 60 days.

Fig. 3 shows the good agreement between the results of agglutination of recipient cells with two different sera. Fig. 4 shows the survival found after two transfusions of blood of different ages. Although the 18-day-old blood was all destroyed in 15 days, it will be seen that the life of the 2-day-old blood was only about 75 days, that is to say, definitely shorter than normal. This patient had in fact hepato-

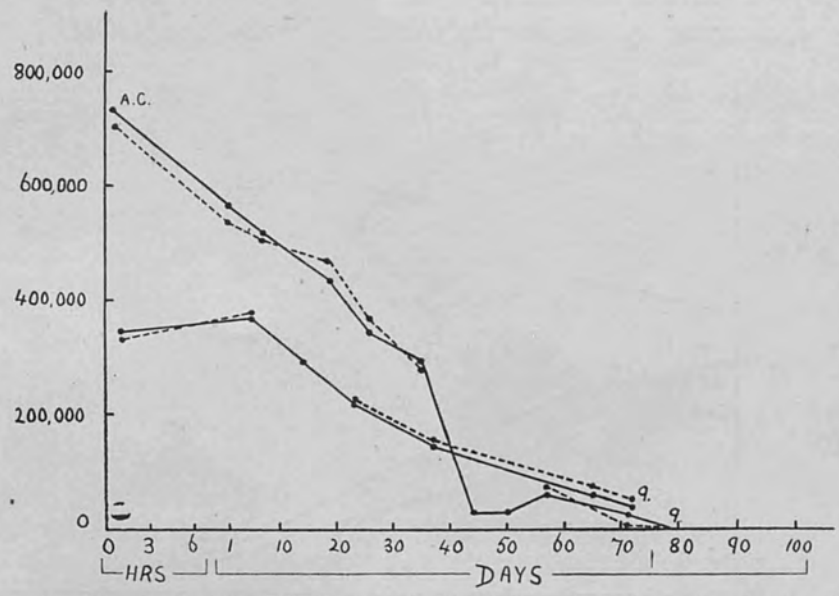

FIG. 3.—Survival of two single transfusions given to different patients. The continuous lines show the figures found when agglutination was performed with sera of the ABO system; dotted lines indicate those found with the MN sera. Note the good agreement. (Patient A. C. was a child.)

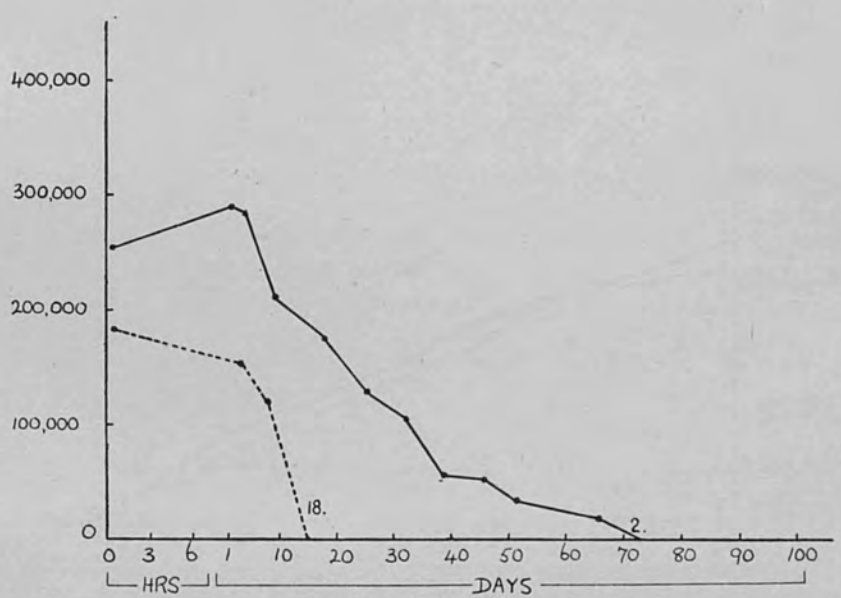

FIG. 4.—Survival of two transfusions given consecutively to the same patient. Note that the survival of the 18-day-old blood is very short, and the survival with 2-day-old blood is definitely less than expected.
The patient was suffering from an undiagnosed variety of reticulosis.

splenomegaly, and was jaundiced before the transfusion. Since in this case the destruction seems to have been more rapid than in the others, it may certainly be rash to draw conclusions about the life of transfused

erythrocytes from a few cases. Very much greater certainty is given to the conclusions by the results in the following section of the work.

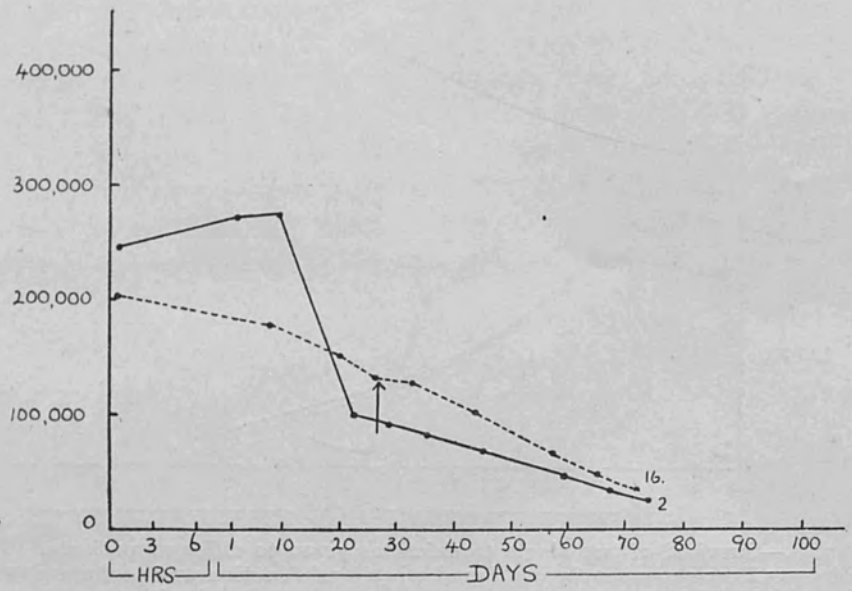

FIG. 5.

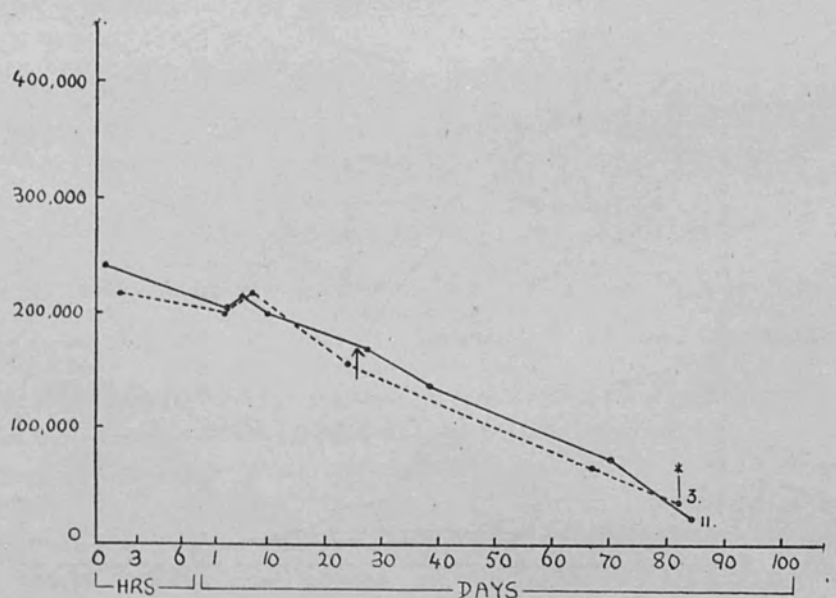

FIG. 6.

Figs. 5–8 illustrate the comparison of the survival of blood of different ages, two simultaneous transfusions having been given in

each case. It will be seen that not only is there little difference between the survival of the different ages of blood, but that the curves suggest

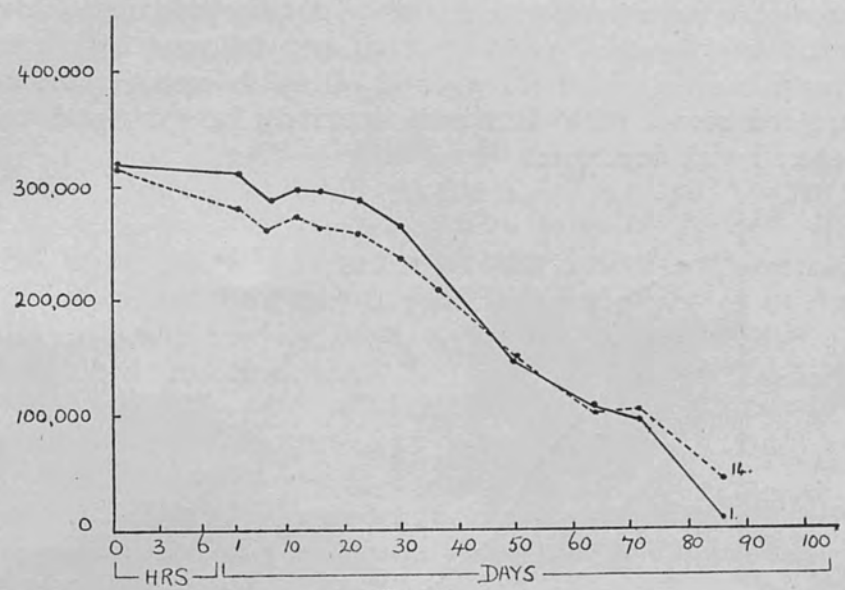

FIG. 7.

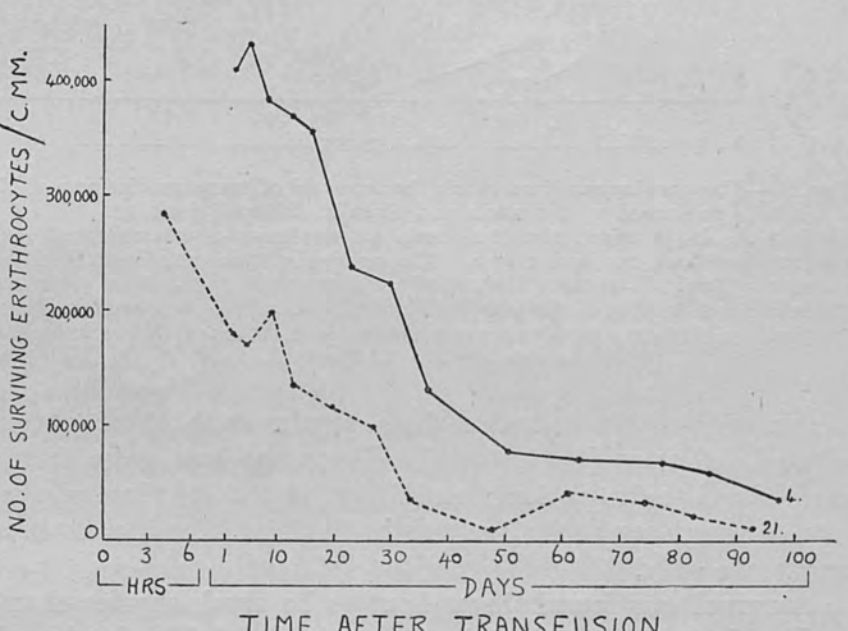

FIG. 8.

FIGS. 5–8.—In each case the figure illustrates the survival of two different transfusions given simultaneously to the same patient. Storage for 16 days appears to have remarkably little effect upon subsequent survival. In fig. 6 the asterisk indicates that a direct test for the donor agglutinogen was positive at this point.

that the life is practically as long as that of fresh blood until blood of over 16 days old is used (see fig. 8).

The importance of the method of storage is emphasised in fig. 9, which shows a comparison between the survival of blood stored with dextrose and blood stored with dextrin. A transfusion of each variety of blood was given to the same patient and followed by the method we have outlined in section (3) (above). It will be seen that the survival of the blood stored with dextrin is definitely inferior to that of the blood stored with dextrose.

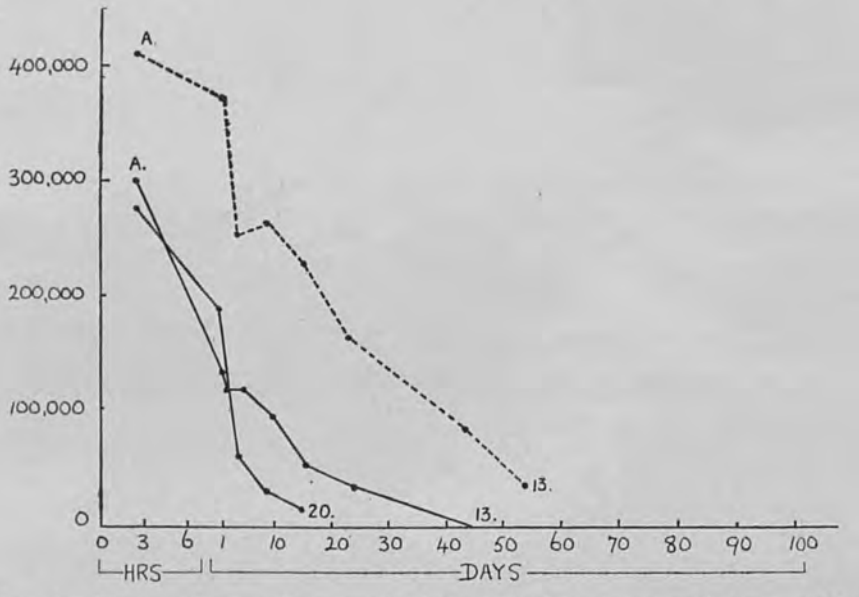

FIG. 9.—The continuous lines mark the survival of two transfusions of blood preserved with dextrin and given to different patients.

Patient A. also received simultaneously a transfusion of blood stored for the same length of time, but in dextrose. The survival of this transfusion is indicated by a dotted line. It is clear that, firstly, the survival of the cells preserved in dextrin is less than that of the cases of blood of equivalent age stored in dextrose, and secondly, that in the direct comparison blood stored with dextrose has an obviously greater survival.

It is interesting to compare these results with those obtained by Wiener and Schaefer [1939, 1940]. These workers gave transfusions of stored blood to 28 patients and followed the survival of the donor cells by a rough quantitative method. The blood was kept with sodium citrate only.

The survival was found throughout to be much shorter than in this work. For instance, two patients received 13-day-old blood. The time for total disappearance of donor cells was found to be 56 days in one and 42 days in the other recipient. The authors suggest that each day of storage reduces the survival by about 6 days.

The results, although showing a general inverse correlation between length of storage and subsequent length of survival, are very variable and give little information about the intermediate "curve of destruction." The authors suggest that the variations are accounted for by

differences in the technique of taking blood and in the patients' treatment of the transfused cells. It seems more probable, however, that they are to be accounted for by the rather rough quantitative method employed.

The greatly inferior survival of the cells of blood stored with citrate compared with that of the cells of blood stored with glucose in addition might have been anticipated from a study of the results of *in vitro* experiments on stored blood. Rous and Turner [1916] first showed the value of glucose as a preservative for red cells, and numerous investigators have confirmed their results in recent years.

Discrepancies in the life of stored blood will probably be reported until the optimal preserving solution and the optimal dilution for the storage of blood are agreed upon.

Summary.

Twenty patients have received 26 transfusions of stored blood, and the fate of the erythrocytes of the donor followed quantitatively by differential agglutination.

A method of performing control agglutinations using the sera both of the ABO and MN systems is suggested.

A further application of differential agglutination is outlined. Its purpose is the following of the fate of two transfusions given simultaneously. This method has been used for (1) comparison of methods of storage; (2) to obtain a comparison of the survival of two different "ages" of blood in the same patient.

Conclusions.

1. A high proportion of the erythrocytes of blood stored in citrate-saline-glucose survive transfusion. Furthermore, the total time of survival is little inferior to that of fresh blood.

2. These observations suggest that the present method of storage is good and that little more than the normal ageing process occurs at least in blood stored for 18 days or less.

In connection with Ashby's work on the survival of the transfused erythrocytes of fresh blood, P. E. Weil [1940] has remarked: "Ce n'est donc pas aux depéns de l'hæmoglobine des globules détruits que l'organisme trouve le possibilité de former des nouveaux globules, mais ce sont bien les globules transfusés eux-même qui continuent leur vie et leurs fonctions physiologiques."

These words might be used as a refutation of the remarks quoted at the beginning of this paper.

APPENDIX.

IN VITRO EXPERIMENT TO DETERMINE ACCURACY OF COUNTING CELLS OF GROUP "ON" IN THE PRESENCE OF CELLS OF GROUP "BM" AFTER AGGLUTINATION WITH (1) BETA SERUM, (2) ANTI-M SERUM.

Description.—A suspension of "ON" cells was prepared and two dilutions of it were made. To each dilution was added an equal volume of a more concentrated suspension (1/50) of "BM" cells. 0·1 c.c. of this solution was mixed (1) with 0·1 c.c. of beta serum, and (2) with 0·1 c.c. of anti-M serum, in small bottles (see description of method in text). After two hours, counts of the free cells were made. In all, 31 counts were performed.

By counting the suspension before mixing, the expected number of free cells could be calculated.

Expected.	Found.	
	With beta serum.	With anti-M serum.
1,134,000	1,195,000 1,200,000 1,189,000 1,218,000 1,189,000 1,188,000 1,189,000 1,248,000	1,195,000 1,227,000 1,179,000 1,138,000 1,139,000 1,198,000 1,195,000
577,000	558,300 565,800 576,700 547,400 554,200 585,000 575,000 570,000	550,800 554,100 545,000 573,300 542,700 556,000 557,500 551,700

By agglutinating with BM cells only, blank values were obtained, viz. (1) with beta serum 20,000 cells per cu. mm. (av.), (2) with anti-M serum 22,000 cells per cu. mm. (av.). These figures have been added to the number of free cells to be expected.

It will be noticed, firstly, that agglutination with beta and anti-M sera give closely similar results. Secondly, it will be seen that the "scatter" of the figures is small, but that in the first dilution all the figures are greater than expected, and in the second dilution they are, with one exception, lower. These errors presumably arise in performing the dilutions and, therefore, are liable to occur in any experiments made with this method.

The coefficient of variation in this experiment was found to be approximately 5 per cent., that is, two counts must differ by at least 10 per cent. to be significantly different.

This figure may be compared with one given by Ponder [1934] (for ordinary blood-counting). According to this author, when 500 cells only are counted, two counts must differ by more than 12 per cent. before the difference can be considered significant.

As only 500 cells were counted on each occasion in this experiment it will be seen that the error is of the same order as that of ordinary blood-counting.

We wish to thank Dr. J. O. Oliver, former Director of this Depot, and Dr. O. M. Solandt, his successor, for advice and criticism, and also Dr. M. Maizels, who provided the impetus for this work and who was generous enough to allow us access to his results at all stages.

We should also like to thank Dr. G. L. Taylor of the Galton Laboratory Serum Unit for providing M and N agglutinating sera, without which this section of the work could not have been done.

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ISOAGGLUTININ CHANGES
AFTER TRANSFUSION OF INCOMPATIBLE BLOOD AND SERUM

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I. MAUREEN YOUNG, B.SC.

(*South-west London Blood-supply Depot*)

LITTLE attention has so far been paid to serological changes in the recipient after the transfusion of incompatible blood. There are only 3 recorded cases in which such changes have been noted.

Rø (1937) reports the case of a patient of group O who received 500 c.cm. of group A blood. Although preliminary cross-matching showed no agglutination of the donor's cells by the recipient's serum, when the test was repeated with serum obtained two days after the transfusion agglutination occurred up to a dilution of 1/20. At the end of five weeks the agglutinating power of the recipient's serum for A cells had increased further and agglutination occurred in a dilution of 1/200. Four weeks later the titre had fallen to 70 (the titre being expressed as the reciprocal of the greatest dilution in which agglutination is noted). Rø suggests that the recipient's serum contained only a hæmolysin for A cells before the transfusion and that the introduction of A cells stimulated the production of an agglutinin. In any case, it seems clear that the agglutinin titre rose rapidly during the period following the transfusion.

Wiener (1941) has published details of 2 cases, both of which had been referred to previously (Wiener 1939). The first patient, of group O, received 500 c.cm. of group B blood. Six hours after the transfusion, which was followed by a rigor and hæmoglobinuria but no serious sequelæ, it was observed that whereas the titre of the anti-A isoagglutinin was 32, that of the anti-B agglutinin was as low as 1. Further estimations were made at short intervals, and it was found that while the titre of the anti-A isoagglutinin remained constant, that of the anti-B agglutinin rose steadily, reaching a peak, at the thirteenth day, of 512. The same figure was found when the serum was re-titrated on the twenty-fourth day after the transfusion. The other case was less closely observed. Six days after the transfusion of incompatible blood (of group AB, the patient belonging to group A), it was found that the titre of the anti-B agglutinin was 128; and eight days later 256. It is concluded that these changes represent an absorption of isoagglutinin by the injected agglutinogen, followed by an immune response to the foreign agglutinogen.

In only one of these 3 cases were frequent titrations of the recipient's serum carried out, and in that observations were only extended up to the twenty-fourth day after transfusion. It may therefore be of interest to record the following case in which, after the transfusion of incompatible blood, the isoagglutinins in the serum of the recipient were repeatedly titrated and the changes were followed for some months. The results obtained are in

substantial agreement with those of Rø and Wiener, but changes in the " other agglutinin " were also found, —i.e., after the transfusion of group B blood to a person of group O not only was the titre of the anti-B agglutinin affected but also that of the anti-A agglutinin. These changes were traced to the use of a serum of group different from that of the patient.

CASE-RECORD

A married woman, aged 32, was admitted to hospital at 1 A.M. on Sept. 16, 1940, half an hour after receiving injuries caused by a high-explosive bomb. The injuries were mainly due to falling masonry and included fractures of both legs. She was found to be " shocked " and was treated with warmth, morphia and the transfusion of blood and plasma. After receiving 540 c.cm. of citrated stored blood 3 days old (equal to 360 c.cm. whole blood) and 250 c.cm. of pooled plasma, she improved sufficiently for the doctors in charge to leave her for the night. Next morning it came to our notice that the bottle of blood that had been given was not of group O, as had been assumed, but of group B. The patient was visited by us for the first time at 10 A.M., some eight hours after the transfusion had been given. She was then in a very poor condition. Her lips and nails were ashen grey and her skin very cold. No pulse was palpable at either wrist or over either brachial artery and no sounds could be heard at the latter site on auscultation. We were told that although no untoward symptoms had occurred during the transfusion, no great improvement had followed ; the patient's condition had deteriorated since, particularly during the last three hours.

After a preliminary intravenous injection of 150 c.cm. of 3% sodium-citrate, 1500 c.cm. of pooled citrated plasma, 100 c.cm. of four-times normal reconstituted serum and 2 pints of citrated blood (group O) were transfused. The clinical condition improved steadily throughout the transfusion and four hours from the beginning of this treatment, when all but the last 500 c.cm. of blood had been given, the blood-pressure was 116/80 mm. Hg and the extremities had become quite warm.

A quarter of an hour after the preliminary intravenous injection of alkali the patient was catheterised and 95 c.cm. of blood-stained urine was withdrawn. The urine was slightly acid to litmus and had probably all been formed many hours previously, since the blood-pressure was still unrecordable at this time. Very many red cells and some leucocytes but no casts were found in the centrifuged deposit of this specimen; the supernatant fluid only contained 0·7% hæmoglobin (Haldane). The patient was catheterised again at 12.45 P.M., when 170 c.cm. of urine was withdrawn ; this was neutral to litmus. On centrifuging, it was apparent that the red colour was due almost entirely to red blood-cells, the supernatant fluid containing only a trace of hæmoglobin as measured by the benzidine test.

A venous sample was first obtained eight hours after the initial transfusion and the findings were : hæmoglobin 66%, serum-bilirubin 2 mg. per 100 c.cm., serum-proteins 4·3%, blood-urea 55 mg. per 100 c.cm. ; blood group O. Microscopical examination of a suspension showed fair-sized agglutinates which were not increased by the addition of anti-A or anti-B serum. A sample of serum was obtained at the same time and titrated with A and B cells from the donors normally used by this laboratory. The technique employed is that

recommended by Taylor and Prior (1938); the end-point is read microscopically and clumps of 3–5 cells are taken as evidence of agglutination. The titre of the anti-A agglutinin was 128 and of the anti-B only 2 (see fig. 1). At 12.45 P.M., after the patient had received 1500 c.cm. citrated plasma and 100 c.cm. of four-times normal serum, a second venous sample was taken and examined. The hæmoglobin had fallen to 42% while the plasma-proteins had risen to 4·7%. The serum-bilirubin was 2·5 mg. per 100 c.cm., and the blood-urea 45 mg. per 100 c.cm. (see fig. 2). Microscopical examination showed rather bigger agglutinates than in the first smear. The titre of the anti-A agglutinin had fallen to 32, while that of the anti-B had risen slightly to 4.

After the further transfusion of two bottles of blood and 150 c.cm. of 3% citrate, another venous sample was obtained (5.20 P.M.). The hæmoglobin was now 49%, serum-bilirubin 1·8 mg. per 100 c.cm., serum-proteins 4·9% and blood-urea 75 mg. per 100 c.cm. The titre of the anti-A agglutinin was 64 and of the anti-B again 4.

From the time when the patient was first seen by us, an attempt was made to measure the fluid intake and the urinary output. During the first twenty-four hours 3000 c.cm. of lemon squash containing 35 g. of potassium citrate was given (as advised by Bushby and others 1940), and 2000 c.cm. of the same mixture was given during the subsequent twenty-four hours. A rather incomplete record of the fluid intake and urinary output was kept, but it was clear that there was a large negative water balance up to the eighth day, although the urinary output never fell to really low levels.

The patient was visited again on the morning of the 17th, some thirty hours after the incompatible transfusion. Her clinical condition was surprisingly good. She was lucid and not in pain. Her skin looked faintly yellow. She had been catheterised at 5 A.M., and 70 c.cm. of urine had been withdrawn. This specimen contained albumin and was neutral to litmus. The sediment contained many red blood-cells but no casts. The supernatant fluid contained only a trace of hæmoglobin. On this occasion the temperature was 99° F., pulse-rate 120 and blood-pressure 116/80 mm. Hg. The blood still contained agglutinates; the blood-urea had risen to 101 mg. per 100 c.cm., the serum-bilirubin to 3·2 mg. per 100 c.cm. and the serum-proteins to 5·1%. The titre of the anti-A isoagglutinin was 32; that of the anti-B agglutinin still 4.

On the 18th the patient was better, although the blood-pressure was 136/102 mm. Hg and the blood-urea 126 mg. per 100 c.cm. The serum-bilirubin had fallen a little (2·7 mg. per 100 c.cm.). A smear of the blood showed only a few small agglutinates. The titre of the anti-B isoagglutinin had risen to 8 and that of the anti-A isoagglutinin to 128. The urine was alkaline to litmus, and the sediment contained a few red blood-cells and pus and epithelial cells, but once more no casts. During the past twenty-four hours she had passed only 10–20 oz. of urine, compared with a fluid intake of 80 oz.

Until the 22nd (the 7th day) the patient appeared to improve steadily, despite a consistently raised diastolic pressure and a steadily mounting blood-urea. During this period agglutinates were found in the blood, but they became progressively smaller and actually were last seen on the 23rd.

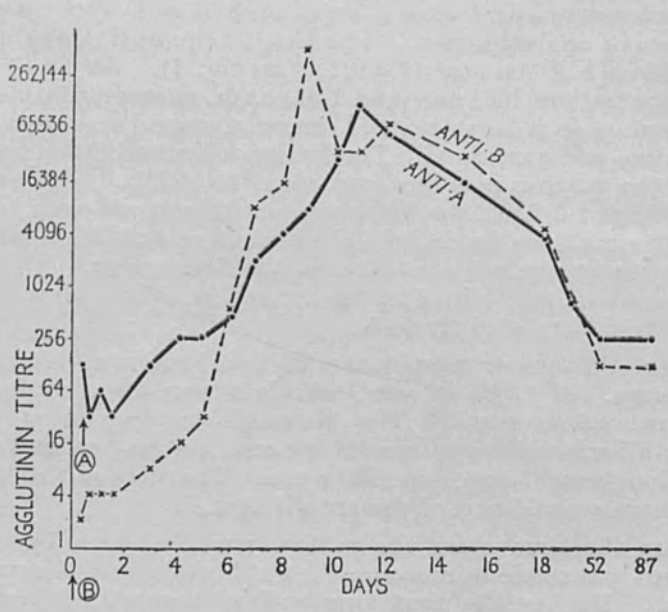

FIG. 1—Changes in titre of serum isoagglutinins after transfusion of (1) group B blood (at B), (2) concentrated serum of group A (at A). Recipient was group O.

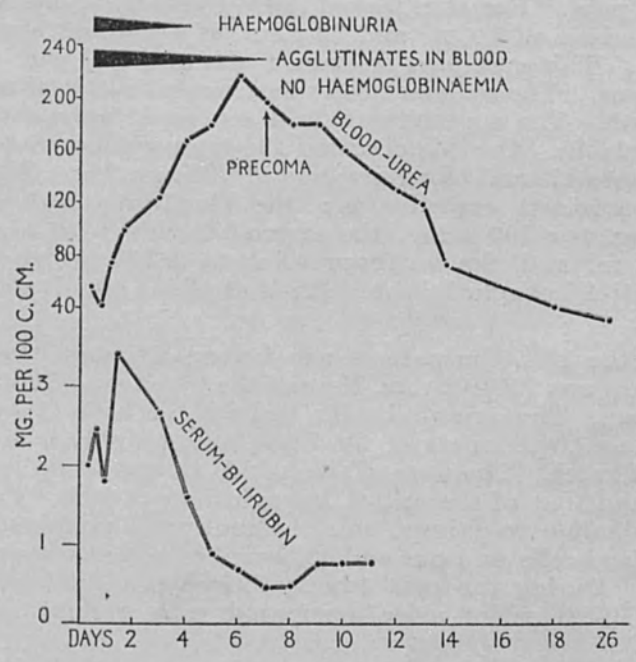

FIG. 2—Findings after incompatible transfusion.

From the 18th onwards the titre of both anti-A and anti-B isoagglutinins rose steadily, and, as will be seen from fig. 1, reached very great heights during the period Sept. 24–27 (9th to 12th days). Between Sept. 23 and Oct. 11, when the titrations were read, it was noted that hæmolysis occurred in the first or first and second dilutions with both A and B cells.

On Sept. 23 the patient was much worse. She was very drowsy, her respirations were deep and there was a heavy brown fur on the tongue; she was considered to be in pre-coma. The blood-pressure was 150/100 mm. Hg. On this and the subsequent two days the patient was made to sweat profusely by giving Dover's powder and aspirin and heating her with electric blankets, &c. Whether as the result of the sweating or not, she was much better on the 24th, and the urinary output increased from this day onwards. On the 25th the improvement was maintained and the blood-pressure had fallen to 136/80 mm. Hg. From this time onwards, recovery was uneventful from the medical point of view.

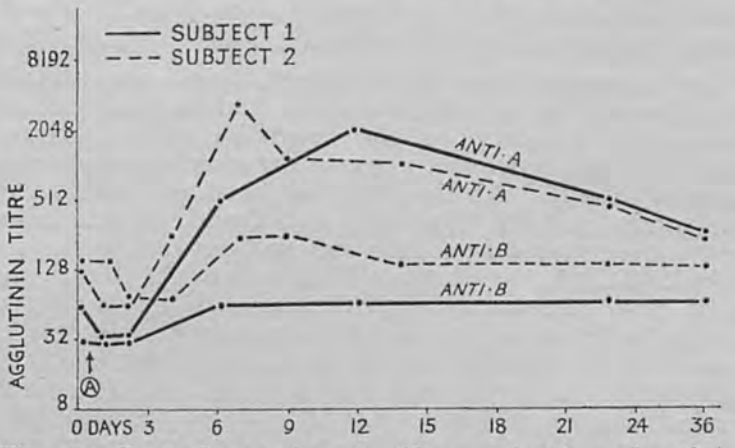

FIG. 3—Control experiments. Changes in titre of anti-A agglutinins in two patients of group O after the transfusion of 100 c.cm. of four-times normal concentrated human serum of group A, reconstituted from the dried product. The serum had been Seitz-filtered before being dried. Transfusions given at A.

During the ten days after the transfusion, two attempts were made to perform renal-function tests. Neither was wholly satisfactory, but both revealed an unequivocal degree of impairment of renal function. On Sept. 21 the clearance was 18% of normal and on the 26th, 30% (van Slyke). On the 26th the urine contained a trace of albumin and a few epithelial and granular casts. The urea-clearance test was repeated on Oct. 4 and was then 50% of normal.

Further examinations of the serum were made during the following months. A slow reduction in the titre of both isoagglutinins was found. From the abnormally high levels reached two weeks after the transfusion the titres gradually fell towards normal.

DISCUSSION

The changes in the titre of the anti-B isoagglutinin will be seen to be of the same nature as those described by Wiener; they consist of an initial reduction, presumably due to an absorption by the transfused group B blood,

followed by a big increase, presumably representing an immune response. The first specimen in our case was obtained eight hours after the transfusion, and thus may be compared with the first specimen in Wiener's case, obtained after 6 hours. The titre of the anti-B isoagglutinin was found, as in Wiener's case, to be extremely low. Thereafter there was a steady rise in titre, and the peak was reached on about the eighth day (not before the thirteenth day in Wiener's case). The extent of the rise, however, seems to have been much greater in this case even when allowance is made for individual differences in the reading of serum titrations. Thus in our case agglutination with B cells could be detected in a dilution of 1/500,000 compared with the figure of 1/512 given by Wiener. Wiener (1941) has recently stated that with the technique that he employs the average isoagglutinin titre is only 16–32. In this laboratory, using the technique referred to above, rather higher figures are obtained (average 64–128). This difference in the normal end-point is insufficient to account for the much greater rise in titre observed in our case. The slow return to a normal level is in accord with Rø's observations.

The changes in the anti-A isoagglutinin—a small initial fall followed by a large rise—at first seemed to be a non-specific response to the injection of the group B blood. Then, however, a more convincing explanation suggested itself. At the time when these observations were made, Aubert, Boorman and Dodd, of this laboratory, were engaged in confirming and extending the work of Schiff (1924); an account of their work is in the press. Schiff first demonstrated the presence of the group-specific substances in soluble form in the serum and postulated an equilibrium between the concentration of agglutinogen in erythrocytes and serum. With this fact in mind, it seemed likely that the rise in the titre of the anti-A agglutinin might have been due to the injection of group A substance in either the plasma or serum that had been transfused in the course of treatment. The plasma, however, was taken from a pool of different groups. It has been demonstrated that this plasma is virtually free of agglutinins, and it may therefore be safely assumed that it only contains traces of the corresponding agglutinogens. The serum, on the other hand, was found to be of group A and had not been pooled. Soon after we had made these observations, Aubert and his colleagues demonstrated that it was possible to produce an immune response in man to the agglutinogen contained in serum of groups A, B or AB. This latter work was carried out with Seitz-filtered human serum. Since the serum injected in our case was a four-times concentrated solution, reconstituted from dried serum * which had been Seitz-filtered before drying, we tried the effect of injecting similarly prepared serum of group A into two people of group O. The results are given in fig. 3. As will be seen, after a small fall, the titre of the anti-A agglutinin rose considerably. Thus a satisfactory explanation for the changes in the titre of the anti-A isoagglutinin in the patient who also received group B blood was reached.

* From the Medical Research Council's serum-drying unit at Cambridge.

It will be noted that the rise in titre of the anti-A isoagglutinin was also very great and considerably exceeds that of the two " controls." It also exceeds that produced by Aubert in any of his experiments (personal communication). The fact that the rise in titre of both agglutinins was so large suggests the possibility of some non-specific stimulation in each case. Aubert has noted slight changes in the anti-A agglutinin after the injection of the B agglutinogen in B serum and vice versa. Further, it is well known that the naturally occurring anti-A or anti-B agglutinin in the serum of some rabbits is often much increased after the injection of OM or ON cells to produce anti-M or anti-N sera. Davidsohn (1938) has pointed out that the isoagglutinin titre of human serum is increased after the induction of serum sickness. In view of these observations it seems reasonable to suppose that some reciprocal non-specific stimulation may have occurred between the one antibody response and the other.

Before this explanation for the changes in titre of the anti-A isoagglutinin had suggested itself, the recipient's serum was tested for the presence of anti-M and N agglutinin, in case their production had been stimulated. No anti-M or anti-N antibodies were found.

As has been mentioned, agglutinates were detected in the recipient's blood-stream up to the seventh day after transfusion. Only until the day after the transfusion, however, were these agglutinates fair-sized. From this day until the seventh only a few small agglutinates persisted, but even on the seventh day a few clumps of five cells were seen. No clumps could be detected on the eighth day or later. Burnham (1930) found some donor cells remaining in the recipient's blood-stream on the third day, but none on the sixth day, after the transfusion of incompatible blood. Wiener and others (1941) in another case found that all but a few of the incompatible cells had been eliminated two days after transfusion.

The rise in serum-bilirubin is a typical finding and is too great to be attributable to the transfusion of stored blood per se. In our experience the transfusion of blood previously stored for three days with a citrate-saline-glucose solution normally leads to only small transient increases in the serum-bilirubin of the recipient.

From the clinical aspect the case adds nothing to existing knowledge of the syndrome of renal insufficiency after the transfusion of incompatible blood, particularly because of the complicating factor of severe injury. As Grant and Reeve (1941) have recently pointed out, there is as yet no precise knowledge of the circumstances in which renal function is disturbed in air-raid casualties.

The passage of many red cells in the urine may have been due to a contusion of the left kidney, since that side of the body was very bruised.

It is interesting to note that there was no immediate reaction to the transfusion—540 c.cm. of citrated stored blood (containing 360 c.cm. whole blood) was transfused in approximately thirty minutes, but no untoward effects were noted. The temperature, however, was not recorded. Others have noted that the transfusion of incompatible blood is not necessarily accompanied by

any obvious immediate reaction (Grove and Crum 1930, Wiener and others 1941).

The progress to uræmia and then to recovery was very similar to that of some of the cases described by Bordley (1931), and the final outcome was in close accord with his observation that no patient receiving less than 350 c.cm. of incompatible blood died.

Knowledge of alterations in the titre of the affected agglutinin, after the transfusion of incompatible blood, has important practical applications. First, if after a hæmolytic reaction, cross-matching is repeated with serum obtained from the recipient within a day or two of transfusion, a false negative result may be obtained owing to the temporary suppression of the agglutinin concerned. Secondly, when the donor cannot be traced, serial titrations of the recipient's serum will decide whether or not the blood given contained an incompatible agglutinogen. Thirdly, cross-matching repeated on the tenth to fourteenth day after transfusion is most likely to detect the presence of a weak or atypical incompatible agglutinin, since the immune response will be maximal at this time.

SUMMARY

After the transfusion of group B blood to a woman of group O, changes in the titre of the anti-B isoagglutinin were observed. There was an initial reduction, followed by a rapid increase, and then a slow fall to the original level. These findings confirm previous observations.

Similar changes in the titre of the anti-A isoagglutinin were also found. These changes almost certainly represent a response to the injection of the A agglutinogen contained, in soluble form, in the group A serum transfused in the course of treatment.

We are grateful to the medical officers of the hospital concerned for allowing us access to their patient and to Dr. O. M. Solandt, then director of this depot, for helpful criticism.

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Subject 4

FAILURE OF IN VITRO TESTS AS A GUIDE TO THE VALUE OF STORED BLOOD

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Many different solutions are still in use for the preservation of blood during storage. This state of affairs seems to be due mainly to the different criteria that are accepted for the comparison of one solution with another. It will be generally admitted, first, that the value of stored blood, contrasted with that of stored plasma or serum, lies chiefly in its content of erythrocytes, and, secondly, that the real worth of these stored erythrocytes must lie in their ability to survive in the recipient's blood stream after transfusion. Nevertheless, most workers have accepted changes occurring in the erythrocytes *in vitro* as an indication of the value of a given method of preservation—in some cases, at least, assuming that such changes are correlated with *in vivo* survival. The changes most commonly measured are alterations in the osmotic fragility of the erythrocytes and the rate at which spontaneous haemolysis occurs during storage.

Criterion of Positive Value of Preservatives

The power to inhibit haemolysis during storage is certainly a desirable property in any preservative solution, since there are limits to the amount of haemolysed blood that may be safely injected into the blood stream. This, however, is a negative property, correlated with safety but not necessarily with value. The only procedure which seems to us to provide a criterion of the positive value of a given preservative solution is to follow the fate of the transfused erythrocytes in the recipient's blood stream. The *in vivo* survival may be measured by the differential agglutination method originally described by Ashby (1919). In brief, this consists in the transfusion of cells of Group O (IV) to a recipient of Group A (II). After transfusion the

recipient's blood contains two types of cells, O and A. The A cells can be " removed " with anti-A serum and the free (O) cells counted. Actually a small fraction of the A cells are not agglutinated, and constitute a " blank " value to be deducted from the total of free cells. By counting the number of donor cells surviving at different intervals of time after transfusion, a sufficiently accurate picture may be formed of the survival of the donor cells in the recipient's blood stream.

Using this method, we have tested eighteen different preservatives in a series of over 100 transfusions of stored blood of various ages. In each case the commonly accepted *in vitro* tests were also applied to the blood immediately before transfusion. Data are presented here which emphasize that these *in vitro* qualities are unsound guides to the actual survival of the same erythrocytes when transfused.

In Table I is set out the constitution of three different preservative solutions. In Table II observations on blood stored for varying lengths of time with these solutions are recorded. The *in vitro* properties of the erythrocytes of a given sample and the corresponding *in vivo* survival of the erythrocytes from the same sample are tabulated.

TABLE I.—*Constitution of the Three Preservatives*

(I) *Citrate - Glucose (Rous - Turner):*	c.cm.
3.8%* sodium citrate	400
5.4% glucose	1,000
Blood	600
(II) *Citrate - Glucose:*†	c.cm.
3% sodium citrate	100
30% glucose	10
Blood	430
(III) *Citrate - Sucrose (Wilbrandt, 1940):*	c.cm.
10% sodium citrate	63
10.3% sucrose	375
Blood	500

Explanation of Methods and of the Terms used in Table II

On the day of transfusion the stored blood to be tested was carefully removed from the refrigerator. The bulk of the supernatant plasma was siphoned off and the remaining cells from two or more bottles of identical age were pooled.

* DeGowin, Harris, and Plass (1940) have pointed out that Rous and Turner used a different salt of sodium citrate from that in present use. The solution employed by us contains a higher percentage of sodium citrate than the original mixture.

† This is a minor modification of the preservative solution recommended in the Medical Research Council's War Memorandum No. 1.

TABLE II.—*Findings with the Three Preservatives described in Table I:* (1) In vitro *Tests.* (2) In vivo *Survival of Cells from the Same Sample. All the Recipients were Women suffering from a Secondary Anaemia*

Time of Storage (Days)	1. In Vitro Tests			2. Survival In Vivo				
					Percentage Survival at Various Intervals of Time			
	Spontaneous Haemolysis (% Total)	Mechanical Fragility (% Free Hb after Shaking)	Osmotic Fragility (M.C.F.) (% NaCl)	Recipient's Age (Years)	4–8 hours (%)	24 hours (%)	1 week (%)	1 month (%)
Blood Stored in Preservative I								
16	0·2	25	0·64	22	99	101	87	70
18	0·4	15	0·74	30	—	96	89	58
21	0·1	29	0·67	28	105	99	94	69
24	0·6	32	0·76	35	85	66	76	52
27	0·3	34	0·72	29	111	84	73	44
28	1·2	34	0·80	38	—	88	65	42
34	0·9	50	0·78	31	84	54	0	0
Blood Stored in Preservative II								
12	0·1	18	0·47	45	112	93	100	71
18	0·2	22	0·51	38	122	88	67	46
19	0·7	32	0·62	30	89	76	73	36
23	2·2	34	0·64	22	—	62	39	13
29	3·0	40	0·68	30	84	39	32	0
Blood Stored in Preservative III								
7	0·3	20	0·27	33	90	93	83	66
9	0·1	28	0·28	73	82	91	75	49
12	0·5	37	0·29	55	80	76	54	0
14	0·6	38	0·37	28	92	32	—	—
16	0·7	40	0·37	—	88	29	—	—

A sample of this concentrated cell suspension was kept for the performance of *in vitro* tests, and the rest was used for transfusion. Three *in vitro* properties were measured:

1. The Degree of Spontaneous Haemolysis—i.e., the amount of free haemoglobin in the plasma expressed as a percentage of the total haemoglobin content of the sample.

2. The Increase in Haemolysis following a Standard Amount of Shaking in the Presence of Glass Beads.—The figures in Table II express the percentage of haemoglobin (Haldane) found in the plasma after shaking a sample of standard concentration. Previous workers have pointed out that the fragility of erythrocytes to shaking increases during storage, but the relationship of this increasing "mechanical fragility" to subsequent *in vivo* survival of stored erythrocytes has not previously been studied. It is interesting to note that Rous and Turner pointed out in 1916 that there is no constant relation between the mechanical and osmotic fragilities of erythrocytes.

3. Fragility to Hypotonic Saline.—In the table only the mean corpuscular fragility (M.C.F.) is recorded—i.e., the percentage NaCl in which 50% of the cells lyse (Dacie and Vaughan, 1938).

After the preliminary withdrawal of a sample of blood from the recipient the transfusion was begun, and was usually completed in two hours or less. A second venous sample was obtained five to ten minutes after the end of the transfusion. Further samples were taken four to eight hours after the transfusion, at twenty-four hours, and thereafter at suitable intervals.

The number of donor cells found in the first sample after transfusion was taken as 100% survival. Subsequent counts were expressed as percentages of this figure. To take this figure as 100% is, of course, not strictly accurate. If the transfusion causes an increase in blood volume, and this is subsequently readjusted (i.e., after the collection of the first sample), the number of donor cells will appear to increase. Conversely, if destruction of erythrocytes has already occurred by the time that this sample is taken, to reckon as 100% the figure found makes survival seem better than it really is. From Table II it will be seen that a sample obtained four to eight hours after the transfusion often shows a relative increase of surviving cells compared with the number found immediately after transfusion and that destruction is dominant with the older bloods only. It may be assumed from this initial relative increase that considerable destruction does not occur during the first few hours after transfusion, except with the older bloods, and the influence of this factor is therefore small.

A further check upon the actual number of donor cells found in the recipient's circulation was made by counting the red cells and measuring the volume of the blood transfused ; then, by taking a figure for the recipient's blood volume based on the known height and weight, the expected concentration of donor cells in the recipient's blood stream could be calculated. In this way it could be demonstrated that approximately the expected number of donor cells appeared in the recipient's blood stream.

Results

The findings set out in Table II are representative of the larger number of samples that have been studied. In order to facilitate comparison the *in vitro* qualities of four samples stored with each of these preservatives have been averaged and set against the average *in vivo* survival of the same samples (arbitrarily taken at one month after transfusion) (see Table III). The ages of the samples stored with

TABLE III

Preservatives	In Vitro Tests			Survival In Vivo
	Spontaneous Haemolysis (% Total)	Mechanical Fragility (% Total)	Osmotic Fragility (M.C.F.) (% NaCl)	Percentage Survival at One Month
I. Citrate–Glucose (Rous–Turner) ..	0·5	38	0·74	54
II. Citrate–Glucose (usual)	1·5	43	0·61	24
III. Citrate–Sucrose (Wilbrandt) ..	0·6	51	0·34	0

In the above table average figures for *in vitro* findings are compared with *in vivo* survival. For this comparison 4 examples of storage with Preservative I (at 18, 21, 24, and 28 days) have been contrasted with 4 examples with Preservative II (at 18, 19, 23, and 29 days). In the case of Preservative III, with which survival is so much inferior, 3 younger samples (12, 14, and 16 days) were chosen for comparison.

Preservatives I and II are comparable, but in the case of Preservative III younger samples have been chosen because of the much poorer survival. Since the cases are representative of a larger series that has been studied, the following conclusions seem justified:

The survival of transfused cells is better after a given period of storage with Preservative I than with Preservative II and is much less good with Preservative III.

The osmotic fragility of stored erythrocytes is no guide to their subsequent *in vivo* survival. It might appear from this small series that there is even an inverse correlation between

osmotic fragility and survival ; findings with other preservatives, however, show that there is no constant relation.

A small amount of haemolysis in a given sample does not necessarily imply good *in vivo* survival—e.g., Preservative III.

Fragility to mechanical trauma is only a very approximate guide to survival.

Other Preservatives

Other anticoagulant solutions studied in this way include heparin, sodium citrate with and without glucose in different concentrations and dilutions, "I.H.T.,"* citrate - dextrin, and combinations of sucrose, glucose, and sodium citrate ; defibrinated blood was also tested. The findings with these other systems support the contention that fragility to hypotonic saline is no guide, and other commonly measured *in vitro* properties are at best a poor guide, to subsequent survival.

On the basis of *in vivo* survival tests the preservative solutions used fall into two main groups: (1) systems containing no glucose ; (2) systems containing glucose.

1. The first group includes heparinized blood, defibrinated blood, blood stored with "I.H.T.," with sodium citrate, and with citrate - sucrose and citrate - dextrin mixtures. After the transfusion of blood previously stored with any of these anticoagulant solutions for less than seven days the erythrocytes are found to survive extremely well—i.e., virtually as well as those of fresh blood. (After a fresh-blood transfusion it is found that the donor cells are slowly and steadily eliminated over a period of approximately 100 days—i.e., about one-third are eliminated during each month after transfusion (Ashby, 1919 ; Martinet, 1938).) Subsequently deterioration is rapid, and after two weeks' storage the majority of the transfused cells are usually eliminated from the recipient's circulation within twenty-four hours of the transfusion. To particularize, blood stored with a large relative volume of 3% sodium citrate falls below the average for this group, whereas blood stored with dextrin survives a little better than the average.

2. In general, blood stored with glucose survives extremely well after transfusion—i.e., virtually as well as fresh blood—when it has been stored for less than fourteen days. (This is a slightly more conservative estimate than one previously made with a similar type of anticoagulant solution (Mollison and Young, 1940).) There is a pro-

* "I.H.T." is the name given to an anticoagulant solution containing 0.5% sodium citrate, 0.7% NaCl, and traces of other salts; this solution has been much used in Russia (Bagdassarov, 1937).

gressive deterioration during the third week of storage, but blood stored with glucose for three weeks is usually not completely eliminated from the recipient's circulation for more than a month after transfusion. These remarks apply to citrate - glucose mixtures in which the volume of preservative solution is small compared with the volume of blood (e.g., Preservative II). Survival was not found to be improved by increasing the concentration of glucose in the same relative volume of preservative solution. By adding a relatively greater volume of 5.4% glucose, however, survival is improved, and when the proportions of Preservative I are reached a clear-cut superiority is demonstrable. Thus after three weeks' storage with Preservative I survival is still almost as good as if the erythrocytes were fresh. It might appear from this that dilution is one of the factors responsible for the superiority of the Rous-Turner preservative solution. However, in the absence of large amounts of glucose, dilution was found to have an unfavourable effect upon survival, and it therefore seems that the problem requires further investigation.

The addition of sucrose to various citrate - glucose preservative solutions has a favourable effect upon the osmotic fragility of the stored erythrocytes but does not significantly influence their subsequent *in vivo* survival.

Practical Application of these Results

Owing to the low protein content of plasma obtained from blood stored with Preservative I it is not expected that this type of preservative will come into general use. In any case, the solution has to be modified if it is to be used directly for transfusion, because of the large amount of citrate it contains. DeGowin and Hardin (1940) have reported very satisfactory results from such a modification. Storage of blood with Preservative II gives adequate preservation for two weeks and has the advantage of providing plasma with a protein content of approximately 5 grammes %. This preservative solution is virtually the same as that at present in use at the Medical Research Council's Blood Supply Depots, and, as has been mentioned above, it does not seem to be improved by increasing the concentration of glucose.

It may be well to call attention to a few instances from the literature in which fallacious conclusions have been drawn from *in vitro* tests, and to emphasize that some of the solutions that have been recommended are not in fact the best for preservation. Thus Rous and Turner (1916)

concluded that sucrose was practically as good a preservative as glucose for human erythrocytes because of its power to inhibit haemolysis during storage. As we have shown, sucrose is an inferior preservative, judged by its failure to improve *in vivo* survival. Maizels and Whittaker (1939) suggested the use of dextrin because it, too, inhibits the haemolysis of blood during storage. *In vivo* survival tests show that the use of dextrin during storage has only a very slight effect on the survival of the subsequently transfused erythrocytes.

In the selection of the optimal citrate - glucose preservative solution, osmotic fragility tests are, as we have shown, positively misleading. Nevertheless, such tests have been adopted as the main criterion of value by many workers (Bushby, Marriott, Kekwick, and Whitby, 1940 ; Denstedt, Osborne, Roche, and Stansfield, 1941 ; Dubash, Clegg, and Vaughan, 1940) ; the preservative solutions recommended by these authors therefore contain only small amounts of glucose.

It is clear that no new preservative solution should be recommended until its effect on the subsequent *in vivo* survival of the erythrocytes has been determined.

Fate of " Fragile " Cells after Transfusion

A corollary of the view that the osmotic fragility of stored erythrocytes is an indication of their ability to survive *in vivo* must be that cells with an increased osmotic fragility soon rupture when introduced into the recipient's blood stream. Such a view has been expressed by Denstedt *et al.* (1941), for example. If this were true there would be two possible findings immediately after the transfusion of blood with a raised osmotic fragility. Either the recipient's blood stream would contain numerous fragile erythrocytes, in which case the osmotic fragility curve of the recipient would be found to be altered, or, if the donor cells had already ruptured, there would be free haemoglobin in the recipient's plasma and *in vivo* survival tests would show far less than the expected number of donor erythrocytes.

In practice neither of these possibilities is observed. On the contrary, first, transfusion of blood with an increased osmotic fragility does not affect the fragility curve of the recipient ; secondly, following the transfusion of such blood, haemoglobinaemia is not observed ; and, further, the expected number of donor cells is found, at least temporarily, in the recipient's blood stream. From this one can only

conclude that the osmotic fragility of the donor cells has undergone a considerable change.

The above statement is based upon observations with preservatives containing glucose. For example, when blood that had been stored for twenty-one days in Preservative I was transfused, the curve of osmotic fragility of erythrocytes taken from the recipient, both before and after the transfusion, was estimated. Although the M.C.F. of the donor erythrocytes had been found to be very high (i.e., 0.78% NaCl) immediately before being used for transfusion, the osmotic fragility curve of samples taken from the recipient before and after the transfusion showed no difference. The transfusion occupied approximately forty-five minutes, and the sample was obtained about five minutes after the transfusion had been completed. Both before and after transfusion, in estimating the osmotic fragility, it was found that haemolysis began in 0.46% saline and that 50% of the cells lysed in 0.39% saline.

The *in vivo* survival test showed that the recipient's blood stream contained approximately 560,000 donor cells per c.mm. (the expected number) immediately after the transfusion, and thus the only possible conclusion is that the donor cells must have undergone a change in osmotic fragility.

This reversal of changes in osmotic fragility may also be studied *in vitro*. For instance, in the case referred to above a sample of the donor cells was placed in fresh compatible plasma for one hour and its osmotic fragility was then re-estimated. It was found that the M.C.F. of the cells had practically reverted to normal (actually 0.45% NaCl). This effect was found to be constant with this particular preservative. It should be pointed out that although the majority of the cells in a given sample succeed in re-acquiring an approximately normal resistance to hypotonic saline, a few may rupture. The most likely explanation of this reversal in osmotic fragility is that glucose is washed out of the cells when they are placed in a solution of relatively high tonicity at room temperature.

Reversal of osmotic fragility was not found to occur with blood stored in certain other preservatives. For instance, when erythrocytes that had been stored with Preservative III were placed in fresh plasma their mean corpuscular fragility did not appear to be affected. This is probably because alterations in the base content of the erythrocytes have occurred and because base is not so mobile as glucose. Nevertheless, the fact that the M.C.F.

of stored erythrocytes can be altered in certain circumstances by allowing them to stand in fresh plasma is a further reason for regarding the osmotic fragility, as usually measured, as being an artificial criterion of value.

Summary

The *in vivo* survival of the erythrocytes of stored blood following transfusion cannot be predicted from a knowledge of the results of the commonly accepted *in vitro* tests made upon the sample. The results of work based upon such *in vitro* tests may therefore be completely fallacious.

Many different preservative solutions have been tested for their effect on the *in vivo* survival of the subsequently transfused erythrocytes. Some details of results obtained with three of these solutions are given and results with the rest are summarized.

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