Comparative studies of flagella from *Proteus* species

by

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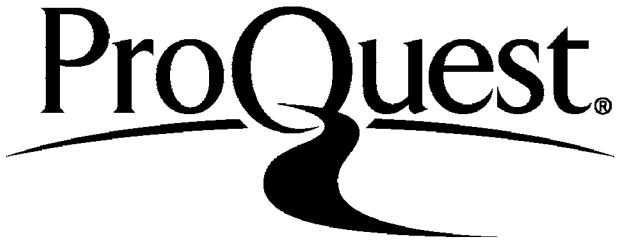


ABSTRACT

Certain aspects of the flagella and flagellar proteins of selected members of the Proteus and Providence groups have been compared.

Proteus flagella, negatively stained and examined in the electron microscope, presented both "beaded" and "lined" surface appearance. Structures which possibly represent cross sections of flagella were observed; these show two groups of three sub-units with a slight gap between the triplets.

Reaggregation of flagellin occurred in the presence of high concentrations of salt.

Purified flagellins of P. vulgaris, P. mirabilis, P. morganii and P. rettgerii and the Providence group gave single bands when electrophoresed in starch or polyacrylamide gels. Comparison of the mobility of purified flagellins revealed great differences, even between those flagellins isolated from strains of a single accepted species. In addition, tryptic peptide maps of purified flagellins each had a distinctive pattern.

Molecular weights of flagellins of the four Proteus species were assessed by electrophoresis in polyacrylamide gels containing SDS; in all cases values of about 40,000 were obtained.

Amino acid analysis revealed the presence of ϵN-methyl lysine and histidine; neither have been reported conclusively in this group before. ϵ N-methyl lysine was restricted to flagellins from certain P. morganii strains while histidine was found in approximately half of the flagellins examined, irrespective of species.

The C terminal amino acid of all the flagellins examined is arginine; the C terminal sequence is ...ser - leu - leu - arg COOH. In every case alanine is the common N terminal amino acid. Seven out of

thirty-three of the peptides found on tryptic peptide maps of P. vulgaris NCTC 100 20 flagellin have been completely or partially sequenced.

The possibilities of using cyanogen bromide to cleave Proteus flagellin were investigated; citraconylation and maleylation were both extremely effective in keeping the resultant cyanogen bromide fragments in solution.

The results are discussed with regard to inter-relationships between the species and the possible uses of these results in the taxonomy of the genus Proteus and the Providence group.

TABLE OF CONTENTS

ABBREVIATIONS

The abbreviations used in the text are those proposed by The Biochemical Journal, "Policy of the journal and instructions to authors", (revised 1972).

Other abbreviations are given in brackets when the full term first occurs. They are:

NCTC	National Collection of Type Cultures
NML	ϵ N-methyl lysine
SDS	Sodium dodecyl sulphate
TFA	Trifluoracetic acid
DPTU	Diphenylthiourea
DNS-Cl	1-dimethylaminonaphthalene - 5-sulphonyl chloride
PTU	Phenyl isothiocyanate
DFP	Diisopropylphosphofluoridate

INTRODUCTION

Bacterial appendages were familiar to early light microscopists from *in vivo* studies with dark field microscopy or by the examination of stained films. The structures they observed consist of bundles of organelles now termed flagella. These flagella may be present at one or both of the poles of the bacterial cell, that is, the cell shows polar flagellation or, in peritrichously flagellated cells, be uniformly distributed over its surface.

A. General morphology of flagella

Bacterial flagella are long, uniformly thin, unbranched filaments which are attached at one end to the bacterial body; they may or may not be surrounded by a sheath. If stained films are examined in the light microscope or with the electron microscope flagella show in plan a sinuous form indicative of an *in vivo* helical form.

This helical form has been demonstrated by Mitani and Iino (1965) in living specimens of *Salmonella abortivoequina*. They have used dark field microscopy to show the alternation of dark and light parts along the helix of the flagella bundles. Using phase contrast microscopy Weibull (1950; 1960) has also observed the helical structure of flagella bundles and showed that in the strains of *Proteus vulgaris* and *Bacillus subtilis*, which he studied, the helix is left handed.

The values for the pitch of the flagella helix, as measured in living material, and the figures for the sinusoidal wavelength obtained from flattened preparations, either stained and examined in the light microscope (Leifson, 1960) or studied in the electron microscope by shadowing or negative contrast methods, show reasonable agreement. In many species the pitch of the flagella helix is constant and lies between about 1.5 and 2.5 μm. A radius of 0.5 μm is usually found

for a flattened helix. The number of pitch lengths is generally between 3 and 6, the overall length of the flagellum being several times that of the bacterial cell. In the extreme case of Spirillum volutans, Reichert (1909) reports a total flagellar length of 72 μm containing 6 pitch lengths.

Occasionally it has been noticed that one or more different shapes of flagella appear in a bacterial strain. The presence of both normal and curly flagella, that is with a wavelength smaller than that of the normal flagella, is the most common. The phenomenon has been called flagellar biplicity (Pijper, 1957; Leifson, 1960). In Salmonella, curly flagella have been demonstrated to appear in a normal population by mutation (Iino, 1962a) while in diphasic strains of the same organism polymorphic alternation of flagella shape is associated with phase variation. (Iino, 1962a; Iino and Mitani, 1966; Iino and Mitani, 1967.) Hoeniger (1965) has noted a change in the wavelength of flagella of Proteus species when the pH of the growth medium is changed.

B. The fine structure of flagella

Flagella diameters have been reported between 120 and 200 Å. (Lowy and Hanson, 1965; Lowy and Spencer, 1968) Details of the fine structure of the flagella of S. typhimurium, which has been studied in the most detail, were first comprehensively described in 1962 by Kerridge et al. Using electron microscopy they observed a hexagonal pattern of globules arranged about 45 Å apart on the surface of the flagella. It must be noted that these structures were not observed unless the flagella had been subjected to physical treatments, such as ultrasonic vibration. The electron microscope investigations of Kerridge et al coupled with the results of early X-ray diffraction investigations by Astbury et al (1955), indicated that flagella consist of fibrils of globular units either arranged parallel to the axis of the organelle or helically arrayed. Earlier

electron microscope studies by Starr and Williams (1952) on an unidentified diptheroid; Labaw and Mosley (1954) on a strain of Brucella bronchisepta, and Marx and Heumann (1962) for Pseudomonas rhodos had described various periodicities much greater than those for the sub-unit arrangement of Salmonella suggested by Kerridge et al (1962). It is now believed that the periodicities noted by these early workers refer to a sheath covering the flagellum rather than the flagellum itself. Astbury et al (1955) also obtained X-ray reflections from Proteus flagella indicating a 410 Å periodicity. At this time it was thought that all reflexions from vertebrate striated muscle could be indexed as orders of a 410 Å repeat. (See Huxley, 1960.) Actin, a component of muscle, is known to be composed of helices of globular units (Hanson and Lowy, 1964).

Lowy and Hanson (1965) and Lowy and Spencer (1968) have, with their accumulated information, provisionally placed flagella into two main categories: those which have structural features suggesting that they contain other components in addition to the globular protein, flagellin, and those which appear to be made only of flagellin. In the first category are placed those flagella either in possession of a banded structure round the flagellum or a more loosely fitting sheath. In the second category are placed flagella that show either a predominantly "beaded" or "lined" surface when examined with the electron microscope. Salmonella flagella are examples of the "beaded" type; Pseudomonas fluorescens and Bacillus pumilus flagella show a predominantly "lined" surface running parallel to the flagellar axis.

a) Banded and sheathed flagella

Close fitting helically wound bands, thickness 25 Å, have been reported in negatively stained preparations of Pseudomonas rhodos (Lowy and Hanson, 1965). A lined structure can sometimes be seen through these bands. Similar structures were also found by these workers in an unknown strain of P. vulgaris. Sheaths on the other hand are found round

many bacterial flagella and can often be seen isolated as a coherent structure from the flagellum; for example in Bacillus brevis (De Robertis and Franchi, 1951). Bladen and Hampp (1964) have reported a sheath around the flagella of Treponema microdentium which itself shows bands with an axial spacing of about 60 Å. The flagella sheath noted for Vibrio metchnikovii (Van Iterson, 1953) has been recently studied in more detail by Glauert, Kerridge and Horne (1963). The latter workers report that in sectional material the sheath has the same structure as the cell wall. Follett and Gordon (1963) claim that the sheath is easily removed by 6M urea or 0.01 N HCl whereas the flagellar filament is resistant to these reagents. On the basis of these findings they suggest, in agreement with Glauert, Kerridge and Horne, that the sheath is an extension of the cell wall. It seems surprising that the flagella filaments were so resistant to the action of urea and acid since flagella of, for example, Salmonella without a sheath, are susceptible. However, Follett and Gordon fixed the flagella with 0.5% v/v formalin before examination and this may have had some effect on the stability of the flagella.

In some sheaths a definite surface structure can be seen with the electron microscope. The sheath of Spirochaeta seems to be clearly distinct from that of Vibrio in that it appears as a component layer of macro-molecules on the surface of the flagella filament (Holt and Canale-Parola, 1968). Electron microscopy of the sheath of Ps. rhodos (Lowy and Hanson, 1965) has shown it to consist of two helices wound in the same direction. The pitch of each helix is about 254 Å. Both aspects of the helix are visible; one more contrasty than the other. If it is assumed that the contrast is greater on the upper side of the flagellum than underneath, then the helices are left-handed. This would agree with the results of Marx and Heumann (1962) who observed two left-

handed helices, pitch 200 Å, in the sheaths of shadowed flagellar preparations of Ps. rhodos. Lowy and Hanson (1965) note that no clear indication of helical structure has been seen in the sheaths of P. vulgaris, when these are present.

b) "Beaded" and "lined" flagella

As a working hypothesis to clarify the situation between beaded and lined flagella, Lowy and Hanson (1965) have suggested that the surface differences seen in the electron microscope reflect differences in the structure of the sub-unit but that the geometrical arrangement of these units is basically the same in all types of flagella. Hence helical connections apparent as oblique rows are more emphasised in "beaded" flagella whereas the interconnections between sub-units appearing as longitudinal rows are more emphasised in "lined" flagella. They have termed the first category the "A" structure and the second the "B" structure.

(i) Beaded flagella

Many of the studies on the possible arrangement of the sub-units in the flagella of this group have been with the genus Salmonella. Burge (1961) has proposed two alternative models for the flagella structure found in the Salmonella type. One is made up of three helically wound strands and the other is a cylinder of seven hexagonally packed strands with six at the periphery and one inside. Kerridge et al (1962), with the additional information of their electron microscopy of Salmonella flagella have proposed two alternative molecular models for the flagellar fibre. In the first model three longitudinal rows of globular units are wound as in Burge's model. In the second model five longitudinal rows of sub-units run parallel to the flagellar axis. In both a transverse section would show a regular pentagonal arrangement of globular units. Such an appearance has been seen in sectional material.

Lowy and Hanson (1965) proposed a modified model to that of Kerridge et al for Salmonella flagella. Their electron microscope studies indicate four or five rows on the surface of negatively stained flagella preparations and to accommodate this observation they have proposed a model in which eight longitudinal rows of helically connected flagellin molecules are aligned in such a way that the number per turn of each helix equals the number of longitudinal rows. The spherical units are close packed to form a shell. In their model the outside diameter of the cylinder is 163 Å, the effective diameter of the space of the core inside the flagellum cylinder is about 60 Å and the longitudinal rows of units are 43 Å apart. The model also agrees better with the data of X-ray diffraction analyses known at the time (Astbury and Weibull, 1949; Astbury, Beighton and Weibull, 1955) than the former models of Kerridge (1962) and Burge (1961).

Using X-ray and optical diffraction studies Champness (1968, 1971) has confirmed the meridional 52 Å periodicity for flagella of Salmonella and obtained a figure of 44 ± 5 Å for the equatorial maximum. This indicates that the units, as proposed by Lowy and Hanson, are ellipsoid and not globular. With low angle diffraction studies he obtained a value of 130 Å for the flagella diameter. However when he examined stained preparations in the electron microscope he obtained figures for the flagella diameter of 140 Å for preparations stained with phosphotungstic acid and 180 Å for those stained with uranyl acetate.

Burge and Draper (1971) have made a more detailed study of the effect of hydration and certain stains commonly used for electron microscope preparations on the equatorial X-ray diffractions of S. typhimurium. Their findings indicate that great care must be taken in the interpretation of results from both X-ray diffraction and electron microscopy. They have found that the basic integrity of the bacterial flagellum as a cylindrical solid structure is maintained over a wider

range of specimen conditions than allows the preservation of secondary and tertiary structure. They suggest there may be some connection between the subtle spacing and intensity changes observed at high angles of diffraction during dehydration and the observation of beaded and lined flagella (although they have yet to observe lined flagella in Salmonella). Their experiments taken as a whole indicate a flagella diameter for Salmonella close to 140 Å, little affected by hydration or staining with uranyl acetate. However it seems likely that phosphotungstate stained hydrated specimens of short flagella show major increases in flagella diameter due to penetration of stain into a "core" (see later). Burge and Draper have stressed that because of the poor quality of the best X-ray patterns from preparations of flagella it is unlikely that determination of molecular structure will be possible from X-ray data alone.

(ii) "Lined" flagella

"Lined" flagella rarely show globular units or helical connections. Ps. fluorescens flagella stained with uranyl acetate show 5 or 6 lines with an outside diameter of the flagellum of about 170 Å (Lowy and Hanson, 1965). In Bacillus subtilis there are 4 or 5 lines and the outside diameter is about 140 Å (Lowy and Hanson, 1965). Based on studies of partially disintegrated preparations, a model has been constructed for the flagellum of B. pumilus in which ovoid sub-units are arranged to form six fibres coiled around a central core (Abram, Vatter and Koffler, 1966; Abram, Koffler and Vatter, 1966). X-ray studies of the flagellum of this organism (Champness and Lowy, 1968) show that as in Salmonella all the reflections in the meridional direction can be indexed as orders of about 52 Å. Champness has noted that the "lined" flagella of B. pumilus and B. subtilis lack the near meridional reflection found on the first layer line present in the S. typhimurium pattern. He has suggested that a "beaded" appearance of the flagellum

could be explained by the presence of a helical groove which would probably be accessible to negative stain and thus be contrasted in the electron microscope.

Interestingly P. vulgaris does not show a surface appearance which can straightforwardly be described as 'beaded' or 'lined'. All flagella observed in cultures of strain NCTC 4175 showed the globular or "A" structure with either 4 or 5 longitudinal rows of globules (Lowy and Hanson, 1965). In contrast, in an unknown strain of P. vulgaris all the flagella showed the "lined" or "B" structure with either 4 lines seen more or less in face view or 5 lines of which 2 appeared in profile.

A 52 Å periodicity can be deduced from the X-ray diffraction pattern of this species (Champness, 1971). In Ps. rhodos "beaded" and "lined" forms of surface structure were found. However, these seemed to correlate with the presence or absence of a sheath - in general "A" structure was found in the absence of a sheath while when a sheath was present the "B" structure was found (Lowy and Hanson, 1965). In B. stearothermophilus more than one kind of surface structure has been seen along a single flagellum.

At frequent and fairly regular intervals (commonly 600 - 800 Å) along many of the "B" type flagella of Ps. fluorescens, one observes short regions in which all the lines disappear. The lines at either side of each interruption are (often) displaced by half a line spacing relative to one another. Lowy and Spencer (1968) refer to this phenomenon as a line shift. It has been pointed out (Dr. A. Klug, cited by Lowy and Spencer) that such a pattern could arise from seeing two sides of the cylindrical shell of sub-units in superposition; the longitudinal rows of sub-units running at a small angle to the cylinder axis. The structure has been termed a large scale sub-unit helix as against the small scale sub-unit helix represented by the helical connections between sub-units

found in "beaded" flagella. Assuming the same underlying geometry for the sub-unit arrangement in "beaded" and "lined" flagella, the longitudinal rows seen in the beaded form should also run at a small angle to the flagella axis.

However, it is by no means certain that the feature, that is, the line shift, is not an artefact. The helical shape of a flagellum will be severely distorted when it flattens on a carbon film. This might result in the destruction of intermolecular bonds and the ordered structure at certain periodically distributed sites (the discontinuities) at which the flagellum could twist about its axis. Such a twist would be detected only if the lines shifted over a non-integral number of periods. (Lowy and Hanson, 1965.)

c) Is there a central core?

Studies with the electron microscope have provided conflicting evidence as to the nature of the central region of flagella. Some workers have found no evidence for a hollow central core (Lowy and Hanson, 1965) while others have interpreted their results as indicating such a region (Kerridge et al, 1962). The latter workers concluded that the flagella of Salmonella are hollow on the basis of the presence of unstained cores in transversely sectioned material positively stained with lead or uranyl acetate and the appearance of a thin line of stain (phosphotungstate) in negatively stained preparations of flagella which had been partly disrupted by sonication. Both the sections and the negatively stained preparations were made from flagella which had been detached from the cell bodies and thoroughly washed with water. However, Claus and Roth (1964) observed unstained cores in sections of flagella of Acetobacter suboxidans still attached to the cell and not washed with water prior to examination. They found that stain only penetrated into the central area when flagella had been subjected to vigorous physical treatment before staining and examination.

Burge and Draper (1971) in their detailed study of the effects of hydration and staining on flagella specimens, have found that phosphotungstate stained hydrated specimens of short flagella show major increases in flagella diameter. The penetration of the stain makes it difficult to reconcile the results of X-ray experiments and electron microscopy except on the basis of a channel (which could be filled with substances easily washed out) or on the possible alternative of an annulus of stain forming an intermediate layer between a central solid core and the outer sub-unit ring. Presumably the short lengths of flagella in preparations allow the axial penetration of phosphotungstate and give rise to considerable swelling of the flagella together with disorientation of the sub-unit arrangement.

Lowy and McDonough (in Lowy, Hanson, Elliot, Millman and McDonough, 1966) have reported that treatment of synthetic flagella like filaments with trypsin results in the appearance of a very large number of finer structures - analogous to a central core? More recently Martinez, Shaper, Lundh, Bernard & Glazer (1972) have digested sheared flagella of S. typhimurium with trypsin and have also obtained thin filaments. These fibres, which appeared to originate from only one end of the flagellar filament, were not dissociated at extremes of pH or upon heating. The amino acid composition of the purified fibres was very different from that of intact Salmonella flagellin, and the fibres did not cross-react with anti-flagellin or anti-flagellar serum. From considerations of the total flagellar mass it seems likely that these filaments represent an association of large tryptic peptides. The presence of an internal core is not ruled out; the peptides might be attached onto some partially digested core.

d) Basal bodies

Strong evidence for the protoplasmic origin of flagella comes from an experiment of Weibull (1953). He removed the cell wall of Bacillus megaterium by treatment with lysozyme and found that the flagella remained attached to the naked protoplast. Electron microscopy (Houwink and van Iterson, 1950) first demonstrated flagella from autolysed cells. The autolysis removed much of the cytoplasm and therefore gave more contrast to reveal basal bodies of about 100 nm diameter. Subsequently basal bodies were recorded for Spirillum rubrum and Vibrio comma (Grace, 1954) and for Vibrio and Spirillum species (Pease, 1956). In Spirillum it was argued that there was in fact a single basal body for the whole flagellar tuft. However, the preparations examined by all the above authors were made on shadowed preparations of cells that were either old or had been allowed to autolyse in order to make them more transparent to the electron beam. The general size of the structures - up to 0.1 to 0.2 μm in diameter - raised the question of whether the structures observed were in fact artefacts. Pijper (1957) and Kerridge (1961) suggested that basal bodies might be a result of cytoplasmic coagulation around flagellar bases. Moreover in S. undula the size of these bodies has been observed to vary within different cells of the same population (Pease, 1956).

Abram, Koffler and Vatter (1965) have made a more detailed study of basal structure and the attachment of flagella in cells of P. vulgaris using ghosts from standard cultures and from special cultures (grown in the cold) that produce long cell forms. They have found the flagellum to arise within the cell from a nearly spherical structure, 11-14 nm in diameter. This structure appears to be associated with the cytoplasmic membrane. They contend that the flagella which they observed with larger bodies 20-70 nm in diameter

associated with their bases consist, at least partly, of fragments of the cytoplasmic membrane folded around a smaller structure (the basal body proper).

Van Iterson, Hoeniger and Nijman van Zanten (1965,1966) have examined the basal bodies of P. mirabilis both by negative staining and sectioning. They used osmotically shocked cells previously treated with penicillin in the hope of loosening the cell wall structure and have obtained a figure for the basal body of P. mirabilis of 25-45 nm. They believe that the value of 11-14 nm given by Abram et al applies only to a part, that is a small knob, of the whole basal structure; shown as a brilliant dot in negatively stained material. Their thin sections seem to indicate that the flagellum between the cell wall and the plasma membrane is usually constricted to form a neck which where it joins the basal body can be distinguished as a small disc or cup - homologous with the structure recorded by Abram, Vatter and Koffler as the basal body?

Abram, Vatter and Koffler (1966) have noted two flagellar structures in Bacillus stearothermophilus 2184. Just beyond the bend of the hook (which is smooth and 120 - 130 Å in diameter) is a marked region 300 - 350 Å in diameter. This region seems to consist of a central filament 100 - 120 Å in diameter surrounded by a mat of fine fibrils (10 - 20 Å in diameter) which are arrayed in a right hand helix round the central filament. Possibly the structure represents disintegration or assembly of the flagellum?

Actual structural changes of the flagellum have been noted near its proximal end. Lowy (1965) has studied in further detail the flagella of Ps. rhodos which (Lowy and Hanson, 1965) can be either unsheathed or sheathed and are generally of the "A" and "B" type respectively. He found that the proximal ends of both "B" sheathed and

unsheathed flagella showed globular unit structure. Sheathed "B" type flagella had a sheathless region at one end which showed globular units and was about 2000 Å long while sheathless "B" form flagella had a comparable region but only 600 Å in length. The portion of "B" type flagella inserted into the cell wall or body is generally found to be relatively straight, bulbous and tapering, compared to the "A" form which generally has a hooked like form at its proximal end. In addition why is the proximal region of the sheathed flagella of Ps. rhodos about three times longer than in the other flagella? The findings of Lowy (1965) have pointed away from the suggestion of Lowy and Hanson (1965) that in this organism, where both "A" and "B" structure appeared to be present along the length of the same flagellum, such a structural transformation might have some relevance to the functioning of the flagellum.

There is evidence that the morphology of the basal region of flagella may be more complex than has been realised. Recently Depamphilis and Adler (1971 a,b) have succeeded in purifying intact flagella from E. coli and B. subtilis in the form of a filament - hook - basal body complex free from detectable cell wall, membrane or cytoplasmic material. The basal body from E. coli is 27 nm in length and consists of four rings 22.5 nm in diameter arranged in two pairs and mounted on a rod. The top pair of rings are connected near their periphery and resemble a closed cylinder. In B. subtilis the basal body resembles that isolated from E. coli except that the top pair of rings is missing. Dimmitt and Simon (1971) have also succeeded in purifying relatively intact flagella from lysates of B. subtilis and find that the presence of the hook structure seems to confer on the flagellum extra stability to thermal denaturation.

e) Theories of flagella motion

Various hypotheses have been put forward to explain the locomotion of flagellated bacteria in terms of the rotation of a helically shaped flagellum (Jarosch, 1964; Doetsch, 1966,1972). Other mechanisms avoiding the necessity of rotation of the flagellum relative to the cell body have also been proposed (Bütschli, 1883; Reichert, 1909). According to the Bütschli-Reichert hypothesis the flagellum is considered to be a cylindrical rod which has a hypothetical line of contraction running helically round its surface. When this line is shortened relative to other lines running parallel to it on the surface of the cylinder, the rod adopts a helical shape. Movement in a definite sequence of the line of contraction around the surface of the cylinder leads to an apparent rotation of the helically shaped flagellum, though it does not rotate relative to the body. Various ways by which this "line of contraction" could occur have been proposed (Lowy and Spencer, 1968).

C. Isolation, purification and chemical properties of flagella

Migula, (1897) first suspected that flagella were composed of protein. Later studies have confirmed this suspicion and now the protein is extensively studied. Flagellar protein is fairly easy to purify and can be obtained in relatively large quantities.

a) Isolation and purification of flagella and flagella protein

Craigie (1931) in a study of the serological reactions of the flagella of S. typhi used centrifugation to detach the flagella from the bacterial body while Gard (1945) in the course of investigation on poliomyelitis virus isolated intact flagella from S. paratyphi var. odense also using centrifugation. Both examined some of the properties of the flagellar suspensions obtained. A more detailed study by

Weibull (1948) examined some of the chemical and physicochemical properties of P. vulgaris flagella purified by repeated centrifugation. His investigations indicated that flagella decomposed at low or high pH values to give sub-units with an estimated molecular weight of 41,000.

Since these early studies many techniques have been developed to obtain flagella in a pure form and a whole body of knowledge on the nature of bacterial flagella protein accumulated.

Kobayashi, Rinker and Koffler (1959) have made cuts of pellets of flagella sedimented by centrifugation to purify the product. Martinez (1963a) has employed anion exchange chromatography to purify flagella using sodium chloride gradients to elute the adsorbed flagella from DEAE cellulose. Flagella of Vibrio parahaemolyticus and V. alginolyticus have been purified by preparative electrophoresis (Shinoda, Miwatani and Fujino, 1970, Miwatani and Shinoda, 1971). Dimmitt and Simon (1971) have purified relatively intact flagella from lysozyme induced bacterial lysates of B. subtilis. The resulting purified flagella are more stable to thermal denaturation than flagella filaments obtained by shearing. These intact isolated flagella, unlike mechanically detached flagella, were relatively stable until heated above 72°, while intact flagella isolated from a mutant of the bacterium, (presumed to differ by a single amino acid substitution in its flagellin) disaggregated at a temperature 14° lower than the wild type. The process seems to be to some extent a function of the composition of the flagellar sub-unit but in addition Dimmitt and Simon have postulated that the presence of a hook structure confers extra stability on a flagellum.

The name flagellin was originally proposed (Astbury et al, 1955) as a general term for the flagellar protein but it has become limited to describe the protein molecules into which the flagella are

disintegrated as a result of disaggregating procedures. Purified flagellar protein has been prepared by various methods, many depending on the finding that flagella can be dissociated into protein molecules by a variety of agents of which the use of low pH, classically at pH 3, has received the most widespread application. (Weibull, 1948; Kobayashi, Rinker and Koffler, 1959.) Koffler, Mallett and Adye (1957) established the criterion of purity of a flagellin preparation as the absence of pH 2 insoluble material.

Other dissociating agents include alkali (Pijper, 1957) detergents, phenol, sonic oscillation (Koffler, Mallett and Adye, 1957), heat (Martinez and Roscenberg, 1964) and compounds capable of breaking H bonds such as urea (Koffler, Mallett and Adye, 1957). It is to be noted that Martinez et al only use some of the dissociating agents named above to dissociate their purified flagella into monomeric form, not as an aid to further purification.

The proteinaceous nature of bacterial flagellin was first conclusively established for the flagella of Proteus vulgaris and Bacillus subtilis by Weibull (1949a). His purest preparations contained at least 98% protein, no phosphorus and only traces of carbohydrate and fat, which were probably impurities. Ada et al (1964) note that most preparations of flagella prepared by centrifugation contain carbohydrate, the bulk of which is precipitated when the flagella are dissociated with acid. It is of interest that an exceptionally tight binding of non-protein substance to the flagellar protein has been reported in the flagella of B. stearothermophilus (Abram and Koffler, 1964) and Spirillum serpens (Martinez, 1963b). Ether extracts of flagellar preparations from an unspecified source released lipid material (Poglazov, 1966).

The refined techniques for protein separation now available, particularly polyacrylamide gel electrophoresis, have demonstrated the necessity of control of the acid disaggregation process of flagellar protein and possibly for the additional purification prior to analysis. Studies with B. subtilis (Frankel, Martinez and Simon, cited in Joys, 1968) have demonstrated that purification of flagella by differential centrifugation followed by acid disaggregation in the presence of salts yielded preparations which showed multiple bands on polyacrylamide electrophoresis. However, additional purification of the flagella by chromatography on DEAE cellulose prior to disaggregation and treatment with acid in the absence of salts resulted in a preparation yielding only one band.

P. vulgaris (Weibull, 1948), S. adelaide (Ada et al, 1963, 1964) and S. typhimurium phase 2 flagella (Kerridge et al, 1962) B. subtilis and S. serpens (Martinez, Brown and Glazer, 1967) all showed one band in the ultracentrifuge. Ammonium sulphate precipitation tests showed a very narrow range of precipitating concentrations for flagellin (Kobayashi et al, 1959) flagellins of B. subtilis and Spirillum serpens (Martinez et al, 1967) and P. vulgaris (Chang, Brown and Glazer, 1969) gave one band when examined by polyacrylamide electrophoresis. The latter authors showed that a mixture of P. vulgaris and B. subtilis flagellins could be readily resolved.

However, Sullivan, Sui, Suzuki, Smith and Koffler (1969) have made a short report that B. pumilus synthesises two clearly related but distinct flagellins. In addition Shinoda, Miwatani and Fukimo (1970) report the existence of two different sub-units in the flagella of V. haemolyticus. The flagella were purified by preparative electrophoresis on Previkon C-870 and the two sub-units (U1 and U2) distinctly separated by hydroxylapatite column chromatography. The sub-units were shown to differ antigenically and in their amino acid composition. Both, however,

have a molecular weight of about 40,000 and are similar in most physicochemical properties. Miwatani and Shinoda (1971) have also isolated the flagellin of V. alginolyticus and found it to be similar in immunological and most physicochemical properties to one of the fragments (U2) isolated from V. haemolyticus flagellin.

b) Molecular weight of flagellin

Weibull (1948, 1950) using sedimentation and diffusion data estimated the molecular weight of acid dissociated flagellins to be about 40,000. Subsequently, Erlander, Koffler and Foster (1960) claimed that the flagellin of P. vulgaris exists as a monomer, molecular weight about 20,000, below pH 3.8 and as the dimer at a pH above 4.5. However, as most of the determinations were performed in solutions of low ionic strength at a pH several units away from the isoelectric point, it is probable that the low value obtained for the molecular weight could be attributed to the non-ideal behaviour resulting from the presence of a large electrical charge on the protein and insufficient electrolyte to swamp out the charge effects.

More recently Chang, Brown and Glazer (1969) have obtained an average molecular weight of 39,000 ± 1,600 for flagellin of the Purdue strain, used by Koffler in his early studies on P. vulgaris flagellin. They performed their sedimentation equilibrium studies in 6M guanidine in the presence of mercaptoethanol, at pH 8.5. Parish and Marchalonis (1970) used electrophoresis in polyacrylamide gels (pH 4.0, 9M urea) at concentrations ranging from 7-14% (w/v) to assess molecular weights. Using this method they report a molecular weight of 41,000 for the flagellin of an specified strain of P. vulgaris. A value of 39,100 ± 1,000 was obtained from ultracentrifuge studies of B. subtilis flagellin when measurements were made with an alkali dissociated preparation in 6M guanidine at pH 8.3 (Martinez, Brown and Glazer, 1967).

In S. typhimurium molecular weight determinations on flagellin preparations obtained by various dissociation procedures have given consistent values of about 40,000. McDonough (1965) using urea treated flagellin obtained a value close to 40,000 from sedimentation and diffusion data and Archibald's approach to equilibrium method. Asakura, Eguchi and Iino (1964) also obtained a value close to 40,000 with ultracentrifugation of acetone dried flagella dissolved in 3 mM phosphate buffer solution, pH 7.2.

Gel chromatography with Sephadex G100 has been used to establish the molecular weights of the two component flagellins, prepared by preparative electrophoresis, proposed for Vibrio haemolyticus (Shinoda et al, 1970). A molecular weight of 40,000 was found for each component.

Hence the average molecular weight of the component flagellin isolated from many flagellate bacteria seems to be of the order of 40,000. However, more recently, Tauschel (1970) on a study with Rhodopseudomonas palustris flagella reported a molecular weight of 15,500 for flagellin molecules examined in the analytical centrifuge and a molecular weight of 93,000 when the determinations were made using polyacrylamide gel electrophoresis. How this data fits in with the above remains to be seen.

c) Chemical composition of flagellin

Flagellins have been associated with the keratin, myosin, epidermin, fibrinogen group of proteins because of architectural similarity revealed by X-ray diffraction analysis (Astbury et al, 1955). It was thought that an axial periodicity of 410 Å, believed to be present in flagella, correlated with the similar periodicity of the actomyosin complex of skeletal muscle. Establishment of the amino

acid composition of some bacterial flagellins has indicated interesting common features among flagellins but some marked differences from the composition of muscle. Most strikingly, cysteine is absent in all the preparations so far examined, in contrast to the contractile proteins responsible for the movement of animals. An early report of the presence of cysteine in the flagellins of P. vulgaris and B. subtilis (Koffler, Kobayashi and Mallett, 1956; Kobayashi, Rinker and Koffler, 1959) was later disclaimed by the same investigators (cited in McDonough, 1965). Tryptophan is also absent, whereas aspartic acid, alanine, and glutamic acid are remarkably abundant. Threonine, glycine, leucine, valine, lysine, serine, isoleucine, arginine, tyrosine, phenylalanine and methionine are also present. Proline and histidine are found in small amounts or are absent. An unusual amino acid, ∈ Nmethyl lysine (NML), has been reported in hydrolysates of several but not all antigenic types of Salmonella flagellins (Ambler and Rees, 1959). A gene has been located which determines the presence or absence of NML in flagellar protein (Stocker, McDonough and Ambler, 1961). Quantitative replacement of NML by lysine is found in strains which do not contain the methylated amino acid (McDonough, 1965).

A detailed analysis of antigenically distinct Salmonella flagellins (McDonough, 1965) has indicated about 380 residues for a flagellin molecule of 40,000 molecular weight. Generally the amino acid compositions of the flagellins examined was similar. All the flagellins had a high proportion of aspartic and glutamic acids (or amides), threonine and alanine. Little tyrosine, phenylalanine, methionine, proline and histidine was detected. No cysteine, cysteic acid or tryptophan was detected. The g... series of Salmonella antigens lacked histidine and had a phenylalanine to tyrosine ratio of 1.4/1. In contrast histidine was present in Salmonella flagellins of 1,2; i; and e... series and the phenylalanine to tyrosine ratio was approximately

the reverse of that encountered in the g... series. Kobayashi et al (1959) and Chang, Brown and Glazer (1969) both report the lack of histidine in the P. vulgaris strains examined and a phenylalanine to tyrosine ratio similar to the g... series of Salmonella flagella. Glossman and Bode (1972) report a similar situation in the flagellin of a strain of P. mirabilis. From this it would seem that the g... series more closely resembled the Proteus flagellins than did the other Salmonella antigens examined.

To assess the degree of similarity or dissimilarity between the H antigenic types of Salmonella examined, McDonough (1965) expressed the overall differences in the amino acid composition as Δ , the sum of the differences, irrespective of sign, in the number of residues for each amino acid per molecule. For this purpose NML was considered as lysine, and ammonia (and therefore transition between acid and amide) was not taken into account. Using this method slight differences in the amino acid composition between cross-reacting antigens were found while conspicuous differences were apparent between serologically unrelated flagellins. From these results it was concluded that the differences in antigenic character are reflections of the difference in amino acid composition of the protein.

Analyses have also been published of flagellins from Vibrio parahaemolyticus (Miwatani, Shinoda and Fujino, 1970) and V. alginolyticus (Miwatani and Shinoda, 1971). Histidine was present in both the flagellins but proline and cysteine were absent. Comparison of the analyses of the U2 fragment of V. haemolyticus and of the flagellin of V. alginolyticus revealed slightly more methionine and serine in the U2 fragment and slightly more arginine in the flagellin of V. alginolyticus. The B. subtilis and S. serpens flagellins examined by Martinez, Brown and Glazer (1967) both contain histidine and proline with slightly more

of both amino acids in B. subtilis. Other slight differences were also apparent.

d) Cleavage of flagellin

Enzymic cleavage of a protein molecule can give a digest which on peptide mapping employing chromatography and electrophoresis (fingerprinting) gives a distinctive pattern characteristic of the protein. Tryptic digestion gives the most reproducible results since trypsin only cleaves protein molecules on the carboxyl side of lysine or arginine residues while other enzymes are less specific.

For example, Hunt and Ingram (Ingram, 1959) have made tryptic digest of samples of human haemoglobin from normal adults and from those suffering from sickle cell anaemia. The resultant peptide mixtures were separated in two dimensions by paper electrophoresis and paper chromatography. Such "fingerprints" showed that the two proteins differed in only one peptide. The sole alteration in this peptide is the replacement of a glutamic acid residue of normal haemoglobin by valine in sickle cell haemoglobin.

Peptide maps revealed thirty separable peptides from tryptic digests of flagellin prepared from the phase 1 form of S. typhimurium, (McDonough, 1962). This has been further refined to identify 33 out of the 36 expected from the number of lysine (including ϵ N-methyl lysine) and arginine residues calculated to be present for a molecular weight of 40,000. Iino (1964) in agreement has distinguished 35 component peptides in Salmonella. Martinez, Brown and Glazer (1967) differentiated 33 peptides in tryptic digests of B. subtilis flagellin and 36 to 39 peptides in digests of Sp. serpens, while 31 ninhydrin positive peptides were found in tryptic digests of P. vulgaris, (Chang, Brown and Glazer, 1969). In all cases the number of arginine, tyrosine and histidine residues known by amino acid analysis was found to be in good agreement with the number of peptides identified by specific reagents (McDonough,

1965; Martinez, Brown and Glazer, 1967; Chang, Brown & Glazer, 1969).

Four serologically mutant forms of the i flagellar antigen of Salmonella were found to differ from the wild type in one peptide out of the thirty identified at the time (McDonough, 1962) while Iino (1964) reported a difference in only 6 peptides from flagella with antigens 1,2 and enx and a difference in one peptide between a curly mutant and its wild type. Martinez et al (1968) found a single peptide difference in fingerprints of tryptic digests of flagellins from two strains of B. subtilis, one with straight flagella. In this mutant peptide alanine was substituted for valine.

Proteins also can be selectively cleaved by agents other than enzymes. One specific method applied to the study of S. adelaide flagellin has used cyanogen bromide to cleave the flagellin at methionine residues (Parish and Ada, 1969). Four fragments (A, B, C and D) were obtained from the flagellin. These were isolated and characterised as follows:

N terminal					C terminal
Fragment	B	A	D	C	
ala ———	met ———	met ———	met ———	leu-leu-leu-arg	
Molecular weight	12,000	18,000	4,500	5,500	

All but one of the eleven NML residues were located in fragment A. The C terminal sequence for the flagellin was -leu-leu-leu-arg. Glossman and Bode (1972) have similarly cleaved flagellin with cyanogen bromide - the flagellin they used was isolated from a strain of P. mirabilis. The three fragments obtained were maleylated to increase their solubility and prevent aggregation. The alignment of the fragments, their molecular weights and C and N terminals are:

N terminal				C terminal
Fragment	P3	P2	P4	
ala ———	met phe ———	met val ———	leu arg	
Molecular weight	12,500	23,500	5,000	

e) Self assembly of flagellin molecules

Ada, Nossal, Pye and Abbott (1963) were the first to report the *in vitro* polymerisation of flagellin to give flagella-like filaments. Reaggregation of flagellin to give flagella is now known to occur under a variety of conditions. The flagella-like filaments formed are often indistinguishable by available criteria from native flagella.

Both Ada, Nossal, Pye and Abbott; Lowy and McDonough (1964) and Wakabayashi, Hotani and Asakura (1969) have reported the *in vitro* polymerisation of *Salmonella* flagellin subsequent to ammonium sulphate fractionation. Asakura, Eguchi and Iino (1964, 1966, 1968) have reported a process characteristic of crystallisation in which it is necessary to seed a flagellin solution before it will polymerise, while Abram and Koffler (1964) have reconstituted monomeric flagellin of *B. pumilus* at various pH values without the addition of seed or high concentrations of salt.

Flagellin polymerised by $(NH_4)_2SO_4$ fractionation often seems indistinguishable from native flagella, as shown by the electron micrographs of Lowy and McDonough (1964). Wakabayashi, Hotani and Asakura (1969) have found in a more detailed study that rapid and complete polymerisation of flagellin was brought about by the addition of F^-, CO_3^{2-}, HPO_4^{2-} and citrate ions to final concentrations higher than 0.3M. If the concentration of salt was increased from 0.5 to 1.2M the average lengths of the filaments decreased and, in addition, some of the short filaments took on a straight form. Wakabayashi and Mitsui (1970) have used X-ray diffraction studies to show that there seems to be no major difference between the structure of normal flagella and these straight flagella.

Uratani, Asakura and Imahori (1972) have investigated the circular dichroism spectra from monomeric and polymeric forms of

flagellins. A large difference was recorded between the forms; this was not due to the fact that the polymer solutions used in the experiment were inevitably more turbid than the monomer solutions. Taking into account these circumstances these workers consider that when flagellin polymerises into flagella filaments, its secondary structure changes to a large extent.

Asakura, Eguchi and Iino (1964) have found that flagellin prepared by acetone treatment seems to be fairly stable in the absence of salt and at neutral pH. If salts are added to the solution a rapid increase in viscosity takes place, but if the solution was previously subjected to high speed centrifugation no appreciable increase in viscosity occurs. Similarly, solutions of Salmonella flagellins prepared by heat treatment remain in a state of supersaturation. If, however, fragmented flagella prepared by sonication are added to a pure monomeric solution, a rapid increase in viscosity takes place. The ends of the added fragments act as nuclei resulting in the rapid formation of long flagellar filaments. A one-to-one correspondence holds between the added nuclei fragments and the fully grown filaments. More recently (1966) these workers have examined the reaggregation of flagellins isolated from various strains of Salmonella and the effect of the addition of seed of one strain to a monomeric solution of flagellin from another strain. Kinetic studies by viscosity measurement and electron microscope observations showed that the seeds added need not necessarily be homologous with the monomer and that the overall rate of polymerisation is largely dependent on the monomer, some polymerising more rapidly than others. It was found that the shape of the reconstituted filament was determined by the nature of the monomer when a small amount of short fragment was used. However, when moderately long filaments of, for example, curly flagella were used to

polymerise normal monomer the newly polymerised filament was curly.

Abram and Koffler (1964) have polymerised acid dissociated flagellin prepared from B. pumilus in the absence of both high salt concentrations and seed. They found the flagellin reassembled into at least three forms, two of which are highly organised in a linear fashion and appear as straight structures and flagella-like filaments. The reaggregation took place when the flagellin monomers were in deionised water or phosphate buffer and proceeded more quickly at 25° than at 4°. The flagella-like filaments obtained closely resembled native flagella in their morphology. Straight structures were observed in the bulk of reaggregated material up to pH 4.9 while as the pH was raised the proportion of the flagella-like filaments, usually seeming to emerge from the straight structures, increased and at pH 5.3 only occasionally could a straight structure be observed. Material reaggregating at pH 5.4 to 6.3 contained only flagella-like filaments which appeared wavy and as long as 25 µm.

Asakura, Eguchi and Iino have found that the normal type of filament was often transformed into the curly type when it was incubated for a long time. The transformed filaments were reversed into the normal type by treatment with low concentrations of pyrophosphate or ATP. From these results it was concluded that the normal type of flagella filament possesses an intrinsic ability to transform between the normal and curly types, depending on external conditions. Asakura and Iino (1972) have found that copolymerised flagellin (from normal, curly and straight flagella) assumed five common stable forms depending on the ratio of the constituent flagellins. These forms are similar to those obtained by homogeneous polymers which are themselves polymorphic.

Polymerisation experiments with antigenically different flagellin monomers and seed segments have shown that growth of the flagella

fragment occurs from just one end. Asakura, Eguchi and Iino (1968) mixed monomer and seed of two antigenic types and allowed these to copolymerise. After treatment with antisera specific for the monomer some filaments were completely labelled while others were unlabelled, apart from a small portion corresponding to the seed.

Fragments of flagella negatively stained and examined in the electron microscope nearly always appear to be asymmetrical in shape. Abram, Koffler and Vatter (1966) found only the distal ends of flagella had a frayed appearance. Further the asymmetric shape was taken up by growing filaments (Asakura, Eguchi and Iino, 1968). These workers have termed the frayed end of each filament taking the shape of a fish tail the T end and the opposite end the H end. They noted that the free ends of seed filaments associated with beads and branches took the shape of the T end; the association therefore being at their H ends? Evidence from specific antisera labelling indicated the T end of the flagellum as the point from which growth takes place. Pye (Australian Biochemical Society, May 1967) has reported that he was unable to polymerise flagellin from monomeric solutions onto the flagella stumps of mechanically deflagellated cells of S. typhimurium. Flagella fragments could be shown by fluorescent antibody techniques to be labelled only at one end. From the combined evidence he concluded that polymerisation occurs only at one end of the growing flagellum and assumed this to be the proximal end.

It must be noted that if polymerisation occurs at the distal end of the flagellum the newly synthesised flagellin molecules must be transported by some means (a central channel or distortion of the flagellin molecules?) to the point of growth, assuming they are not released into the external medium. Interestingly Iino, Suzuki and Yamaguchi (1970) have reported that exogenous flagellin monomers can be

reconstructed to flagellar filaments at the tip of flagella attached to the living cells, and that the reconstituted flagella on the body can exert their locomotive function.

Effect of breakdown of the molecule on polymerisation

What constitutes the minimal portion of a flagellin molecule that is capable of reaggregation? Which are the binding sites and what is the nature of the intermolecular forces in the flagellum? In the hope of clarifying this point Bode and Glossman (1970) have modified P. mirabilis flagellin in several ways and examined its ability to polymerise. They have found that treatment with carboxypeptidase B completely prevents polymerisation of a seeded flagellin solution. In order to check whether the enzyme treatment affected the flagellin structure they compared the circular dichroism spectra of the untreated and treated monomer. No conformational change was detected. The effect of the enzyme on the flagellin may, however, be more discrete and involve something more than the removal of a few C terminal residues.

D. Growth of flagella in vivo

Although flagellins have been shown to reaggregate in vitro (discussed above) it has been suggested that, in vivo, aggregation is mediated by a biological organising principle. Certain evidence makes it hard to envisage the final step in the production of flagella, that is the association of flagellin molecules, as a spontaneous phenomenon. Some regulatory mechanism must be present in the cell. For example, a non-flagellate mutant of S. abortus equi was found to produce flagellin which can polymerise in vitro but not in vivo. Consequently, the mutant was inferred to be deficient in the initiation of polymerisation in vivo (Iino and Enomoto, 1966; Suzuki and Iino, 1966).

Since, as discussed earlier, polymerisation of flagellin molecules occurs at a structurally defined end corresponding to the distal end of

a flagellum, if flagellar growth *in vivo* is homologous with that *in vitro* it must take place at the tip. In a study of the process of reappearance of flagellate bacteria in deflagellated suspensions of *Salmonella* cells Stocker and Campbell (1959) observed that a flagellum gradually elongates for a limited period and thereafter persists without further elongation. In addition the rate of growth of flagella is unaffected by deflagellation. They observed that up to one generation after deflagellation the mean number of flagella per bacterium was less than before deflagellation, and inferred that a portion of the original flagella forming apparatus was damaged and could not regenerate flagella any more. Kerridge (1961) observed the same phenomenon and proposed two alternative explanations: either the flagella-forming system has a limited life, or that the synthesising system is liable to be damaged as a result of ripping out the flagellum.

The morphological evidence for the existence of a basal granule possibly analogous to the above mentioned biological organising principle is conflicting and has been discussed above. Martinez (1963b) has presented preliminary evidence that the hook structure at the base of a flagellum contains ribonucleic acid. The structure would then be analogous to the flagellosome proposed by Iino and Lederberg (1964) and the flagella-forming system of Kerridge (1960). Iino (1964) proposed a model to resolve the question of the absence of a detectable flagellin pool in *S. typhimurium* (Kerridge, 1963) in which the ribosomes are associated with the basal body and that flagellin is incorporated into the growing flagellum immediately it comes off the ribosome. Martinez now thinks it more likely that the ribonucleic acid concerned attaches to the cell membrane near hooks rather than to hooks themselves (cited in Iino, 1969). Association of basal bodies with tellurite reduction (van Iterson and Leene, 1964) was the result of artefacts produced in preparation for electron microscopy (Abram, Vatter & Koffler, 1966;

van Iterson et al., 1966).

E. Antigenic specificity

Bacterial antigens can be divided into two main categories termed O and H. These seemingly inapt designations for somatic and flagellar antigens were derived from the original study of the distinctive types of agglutination reaction displayed by motile (flagellated) and non-motile (non-flagellated) strains of Proteus. Motile strains of this organism grow out from a point of innoculation on the surface of a solid medium to form a spreading film (German = hauch = H) of growth; non-motile strains grow as discrete colonies (= no film - ohne hauch = O) (Weil and Felix, 1917). There is now a mass of evidence indicating that the H antigens of bacteria are associated with the flagella and the O antigens with the bacterial bodies. One early conclusive study was made by Craigie (1931). He found that removal of flagella by shaking renders cells non-agglutinable by H antisera, and that purified flagella are agglutinated by H antisera but not by O antisera. More recently Nakaya, Uchida and Fukumi (1952) demonstrated that the antiserum for antigen sub-unit g or m of Salmonella enteritidis agglutinated all isolated flagella of this bacterium.

The specificity of the flagellar antigen type has been extensively surveyed in Salmonella and an abundance of well-determined types classified in the Kauffman-White scheme (1965). This scheme also covers antigenic studies on Proteus and Escherichia coli. However, there are few detailed reports of antigenic studies of flagella of bacteria other than those belonging to the Enterobacteriaceae. Antigenic specificity has been noted for the isolated flagella of Spirillum serpens and of different strains of B. subtilis (Martinez, Brown and Glazer, 1967). The strains of B. subtilis showed no cross reactivity. Shinoda et al (1970) have shown that the two flagellins (U1 and U2) isolated from

V. parahaemolyticus by hydroxylapatite chromatography are antigenically different. Miwatani and Shinoda (1971) have demonstrated that the flagellin of V. alginolyticus is antigenically similar to the U2 flagellin of V. parahaemolyticus.

Both the flagellin and its monomer have antigenicity. Ada, Nossal, Pye and Abbot (1963) have shown that the flagella antigens of S. adelaide can exist in three physical states: as a monomer, as an organised (reaggregated) polymer and as the native flagellum. Gel diffusion and immunoelectrophoresis tests showed one main antigen with sometimes evidence of a minor antigen. Martinez et al (1967) working with the flagellins of one strain of Sp. serpens and several strains of B. subtilis detected only one precipitin band when homologous flagellin was used as antigen against flagellin antiserum. However two lines were seen when flagella were used as antigen, one showing identity with the homologous flagellin line. The second line obtained was shown to be homologous to the line obtained if the supernatant of centrifuged flagellar suspensions was used as antigen. It is to be noted that these workers used a different purification technique to Ada for the preparation of the purified flagella.

Though, as noted above, the three physical states show little or no serological differences in vitro, monomeric flagellin differs markedly from flagella in its capacity to cause antibody formation in vivo. Flagella are more effective in exciting a response than polymer which is itself more effective than monomer in inducing antibody formation. Joys (1968) cites the results of Simon, who found that rabbits immunised with flagella from B. subtilis produced 19S antibody which did not react with homologous flagellin. However 7S antibody resulting from hyperimmunisation showed activity on both flagella and flagellin, with a higher titre on the former. Sera prepared against the flagellin, bound to methylated albumin for immunisation, hardly reacted with

flagella, but flagella were able to partially inhibit the antiflagellin/flagellin reactions. Joys concludes from these results that a flagellum and its component flagellin contain common sites of antigen specificity and sites of difference. The flagellin sites could be those masked from the surface when the flagellin molecules are packed in the flagellum or could arise by alteration in the structure of flagellin during its preparation. Such alteration in structure might result in loss of flagellum specific sites, which could also be lost with removal of structural components in the isolation and purification of flagellin.

Precipitation-inhibition tests with the polypeptides obtained by breaking flagellin with cyanogen bromide further indicated that the polypeptide region responsible for antigenic specificity is localised on the flagellin molecule (Parish and Ada, 1969; Parish, Wistar and Ada, 1969). A fragment (A), molecular weight 18,000, has been isolated by these workers and shown to possess all the antigenic determinants of the flagellin molecule. Ichiki and Parish (1972) have partially cleaved S. adelaide flagellin with trypsin and pepsin at sub-optimal temperatures for digestion (25°). Serological tests were used to determine the antigenic activity of the tryptic and peptic digests. These tests led to the conclusion that the antigenic determinants of the flagellin were localised in the pepsin and trypsin resistant fragments of the molecule. Some antigenic determinants were weakened or destroyed by enzymic digestion but the digests could still inhibit to about 90%. The polypeptides, like the cyanogen bromide fragment (A) have in vivo immunological properties which profoundly differ from flagellin.

Martinez et al (1972) have digested intact flagella of S. typhimurium strains SL 870 and SL 871, with trypsin. Thin fibres, which appeared to originate from only one end of the flagellum, were formed upon trypsin

digestion. Flagella and flagellin were found to form a common precipitin band with antiflagellar antibody whereas the fibres did not form a band with the antiserum.

Mutants of S. typhimurium with serologically altered i antigens have been isolated (Joys, 1961; Joys and Stocker, 1966) and shown to possess chemically altered flagellin molecules, as noted by differences in tryptic maps (McDonough, 1962). These mutants possessed new unique specificities and retained some of the original wild type specificities. More recently Yamaguchi and Iino (1969, 1970) have produced antigenic recombinants within the H1 structural gene for flagellar protein by phage mediated P22 transduction. They used non-flagellate mutants of g... complex antigenic derivatives of a stable phase one strain of S. abortusequi. Tryptic peptide maps (1970) revealed that the flagellin of an antigen recombinant was a recombinant of the two parental flagellins, the fingerprint being made up of peptide spots from each of the parents.

Methylation of lysine in flagellin has been shown to be serologically important in certain cases (Stocker, McDonough and Ambler, 1961). In the phase 2 complex 1,2... of Salmonella a correlation exists between NML positive and antigen 3 as when 1,2 type NML negative cells are changed to NML positive by the introduction of the nml gene, antigen 3 appears as well as 1,2 and vice versa (Joys and Stocker, unpublished, cited in Pearce, 1965).

F. Genetics of Flagella

The genetic control of flagellation in bacteria has been studied most extensively in Salmonella using transductional analysis. This genus exhibits a remarkable variation of antigenicity, not only among the different serotypes but also intraclonally. Andrewes (1922) first noted this intraclonal, or phase variation, which is characterised by the appearance of two alternative types of flagellar antigen in a bacterial

clone. Stocker et al (1953) demonstrated that two sets of multiple alleles were involved; Iino and Lederberg (1964) have redefined these sets of alleles as the structural genes for the phase 1 and the phase 2 flagellins respectively.

It has been shown that phase variation results from oscillation of the H2 locus between the active and inactive states (Lederberg and Iino, 1956). H2, when active, is epistatic to H1 and suppresses the formation of phase 1 flagella. When H2 is inactive phase 1 flagella are found. More detailed experiments have indicated (Iino, 1962a, 1964) that each H locus is linked to a specific gene termed its activity controller - ah1 and ah2 for the two H1 and H2 loci respectively. Mutation of $ah1^+$ to give $ah1^-$ results in a strain which is unable to produce phase 1 flagella when the H2 locus is inactive. Mutation of $ah2^+$ to $ah2^-$ results in a loss of ability to produce phase 2 flagella and also in fixation of the H2 locus in its inactive state. As a result the mutant is monophasic in phase 1 (Iino, 1964). Another factor found to control the state of the H2 locus has been termed vh2 (Iino and Lederberg, 1964). Replacement of $vh2^+$ by $vh2^-$ causes stabilisation of the H2 locus in whatever state it was at the time of the mutation (Lederberg and Iino, 1956). Several working hypotheses have been proposed for the mechanism of regulation of the H2 state by the vh2 - ah2 system and are reviewed by Iino (1969).

There is a reasonable amount of data to support the hypothesis that phylogenetically the H2 gene originated by the duplication of H1 . H1 and H2 states are not found in all Salmonellae; many serotypes with antigens of the g... series are deficient in an H2 locus, others have inactivating mutations fixing H2 in the active state. In addition H1 deficient strains and inactivating mutations within the H1 locus have not been described. Very occasionally an H2 gene can be replaced by H1

as a result of unequal recombination and partial structural homology between H1 and H2 has been suggested (Iino and Lederberg, 1964). Occasionally Salmonella strains having three or four phases are isolated from nature (Edwards, Sazaki and Kato, 1962) and in one such triphasic strain duplication of the H1 locus has been demonstrated. Finally, E. coli possesses a single H locus allelic to H1 of S. abony but does not carry an allele of H2 (Mäkelä, 1964).

The mutations causing curly flagella have been shown to be phase specific and closely linked to the H gene of the affected phase (Iino, 1962b). It has since been shown that the site of mutation was inside the H locus (Enomoto and Iino, 1966).

Besides the phase specific regulator genes, a group of phase non specific regulator genes called fla have been found to regulate flagella formation. Mutation of any of the fla genes from fla^+ to fla^- results in the loss of ability to produce flagella in both phase 1 and phase 2. Three groups of fla genes have been recognised. One group is co-transducible with the H1 locus and has been divided into six cistrons on the basis of complementation tests using abortive transduction (A, B, C, D, E and J), (Joys and Stocker, 1965; Iino and Enomoto, 1966). The two other groups are termed fla F and fla G and have only been described in S. abortusequi. Only one mutant in the fla G locus has been isolated and this differs from all other fla^- mutants studied, being the only one to produce flagellin but not flagella.

Non-motile mutants have been described which possess the normal number of flagella per cell and produce flagella which do not differ from those of the wild type strain in either antigenicity, appearance in the electron microscope or configuration as deducted by X-ray diffraction (Enomoto, 1966a). These mutations causing paralysis have been localised in genes termed mot (Iino, 1958, cited in Enomoto, 1966a). Three

complementation groups have been recognised (Enomoto, 1966a, 1966b). Mot C is linked to H1 while A and B adjoin each other and are unlinked to H1.

Joys (1968) has published the following plan of linkage relations of the genes concerned with flagellation in Salmonella:

fla J - fla B - fla D - fla C - fla A - mot C - H1 (including curly) - ah1 - nml

and H2 - ah2 vh2

Genetic regulation in other bacteria

Very little information is available of the genetic control of flagellation in genera other than in Salmonella. E. coli has been shown to possess a single H locus (Furness, 1958), a fla locus (Ørskov and Ørskov, 1962) and a mot locus (Armstrong and Adler, 1967) which may be divisible into two groups, probably adjoining each other. Although allelism tests between the mot genes of Salmonella and E. coli have not yet been carried out, it is most likely that the groups I and II of E. coli are homologous with mot A and mot B of Salmonella. Coetzee (1963) has carried out a preliminary genetic analysis of flagella production and the swarming phenomenon in P. mirabilis, However, this was limited to a preliminary study because of the narrow range of host specificities of the transducing phages available. The swarming phenomenon (Lominski and Lendrum, 1947) seems to be a negative chemotactic response, possibly to a volatile metabolite? Coetzee (1963) found that a phage able to transduce a streptomycin resistance marker in P. mirabilis could also separately transduce the swarming characteristic between variants of two strains of P. mirabilis. A distinction was made between the flagellation locus (or loci) and the swarming locus, which was found to consist of at least three sites. It was not possible to determine whether the swarming and flagella loci were linked. Through lack of non-motile flagellated (paralysed) variants, separate loci controlling the presence of flagella

and their motility conferring properties could not be identified. A modifier gene z was also described which altered the appearance of the swarm sheet - it seems likely that this gene exerts its effect on the growth cycle rather than directly on flagellation.

G. Classification methods

Great reliance is placed on the use of morphological and biochemical characteristics to classify bacteria. However, numerous other methods are now available to assess the position of any one organism in the plant and animal kingdoms and these are increasingly used as an aid in bacterial taxonomy. They include computer aided numerical taxonomy, nucleic acid analysis and hybridisation, genetic analysis by episomal transfer in bacteria, immunological procedures, the study of macromolecular aggregates and sub-unit associations and comparison of the amino acid sequences in homologous proteins.

Comparisons based on amino acid composition differences are difficult to interpret, and reliable data are difficult to obtain. The accuracy of amino acid analysis is insufficient to distinguish between proteins which differ in only 1-2% of their amino acid compositions, while the differences in amino acid composition between proteins which differ in more than 40% of their amino acid compositions are likely to be nearly as great as the composition differences between randomly selected proteins (Ambler, 1968). Peptide maps are satisfactory for comparing very similar proteins or for detecting the results of some one step mutations but are less valuable where large differences are involved.

Many of the comparative studies of proteins take the form of sequence analysis. The greatest insights into the classification of bacteria are likely to come from comparisons of the primary structures of proteins. At this level there is sufficient discriminatory capacity to recognise

both the variant and invariant regions.

When there is other supporting evidence it is possible to construct evolutionary trees using inferred ancestral sequences and evidence from sequence studies. For example, in the animal kingdom classical comparative methods and a reasonable fossil record allow considerable confidence in the suggested relationships of many groups, for example the vertebrates. Zuckerkandl and Pauling (1962) have calculated, from a comparison of the geological record with the number of amino acid substitutions occuring between the various chains of human and horse heart haemoglobin, that an average of 11 million years elapses for every change in an amino acid.

However, plants do not have an adequate fossil record, since by the Upper Cretaceous, when flowering plant fossils became abundant, many of the present day orders were already represented. Similarly, bacteria have no real fossil record. Phylogenetic trees however can be established solely on the basis of amino acid sequence data. The number and nature of differences in amino acid residues are found and from this a matrix of the minimum mutational distance differences is derived.

A protein molecule chosen for sequence studies must be present in many of the major groups of living organisms and be of relatively small molecular weight. Cytochrome c fits these requirements admirably since it is ubiquitous in aerobic organisms and its molecular weight of approximately 12,500 means that progress in sequencing is reasonably rapid. The sequence of this protein has now been established in various plants, animals, fungi and bacteria. The amino acid sequence of horse heart cytochrome was the first to be reported (Margoliash, Smith, Kreil and Tuppy, 1961).

Since then sequences have been established for Tuna fish (Kreil, 1965) and a whole range of other animal cytochrome c's (see Dayhoff, 1969).

Heller and Smith (1966) established the sequence of Neurospora cytochrome and more recently several plant cytochromes have been sequenced by Boulter and his colleagues: Ginkgo biloba L. (Ramshaw, Richardson and Boulter, 1971); Brassica napus L. (Richardson, Ramshaw and Boulter, 1971) and Helianthus annus L. (Ramshaw, Thompson and Boulter, 1970).

It has been established that 35 invariant residues are common to plants, fungi and animals. All this is strong presumptive evidence that plants, animals and fungi derive from a common ancestral form and that the cytochrome c structural gene has survived as a distinct recognisable entity for over 2 billion years. Bacteria, however, do not appear to contain a cytochrome of the same homologous series. Ambler (1963a, b) has sequenced cytochrome 551 of Pseudomonas fluorescens and found it to show considerable differences from the cytochrome c of animals, plants and fungi already studied. The cytochrome 551 of Pseudomonas fluorescens does show some homology in that there is similarity in charge distribution between the N terminal portion; residues 1-20 of this cytochrome are similar to horse heart cytochrome c. Hence it is possible that the bacteria diverged from the main stock very early in evolutionary history.

Few studies of the primary sequence of the bacterial proteins are known. Bruschi and Le Gall (1972) have reported the primary structure of cytochrome c 553 of Desulfovibrio vulgaris. Crawford and Yanofsky (1971) have compared the sequences of the first 50 residues of the α chains of the tryptophan synthetase of E. coli and Pseudomonas putida. They have found 50% identity of the residues; most of the residues which differ are chemically dissimilar and half of them are specified by codons which differ by more than a single base. The two residues known by mutational analysis to be essential for catalysis in E. coli are preserved in Pseudomonas putida. Work has begun on the sequences studies of the

rubredoxins (Benson et al, 1971). If these studies on the proteins sequenced to date can be extended to other species, and also include other bacterial proteins, much information should be provided on evolutionary relationships within the bacteria, since to be believed, results based on sequence studies of one protein must be found consistently with a number of proteins of different types and functions.

H. The classification of the genus Proteus

Various classificatory schemes have been proposed for the group Proteus-Providencia (Rustigian and Stuart, 1945; Kauffman, 1954, 1959; Breed et al, 1957; Ewing, 1958; Romanenko and Raginskaya, 1971). Some scientists think that it is incorrect to link the biochemically related species, P. vulgaris and P. mirabilis to independent species of the same genus. Kauffman (1964) has suggested that these two species should be grouped into one genus, naming it P. hauseri.

The correctness of including P. morganii in the genus Proteus has long been disputed. Thus, the Russian school of microbiologists has considered these bacteria as micro-organisms with no relationship to the Proteuses (Tarasova, 1940; Christivich, 1942; Ivanova, 1956). At the same time Breed et al (1957) distinguished P. morganii as an independent species of the genus Proteus.

Studies of the percentage of guanine plus plus cytosine in the DNA of bacteria from the above group have shown P. morganii to have a guanine + cytosine content of 52%, while P. vulgaris, P. mirabilis and P. rettgerii and the Providence strains examined have 40% guanine plus cytosine in their DNA (Falkow, Ryman and Washington, 1962). The percentage of guanine plus cytosine recorded in the Salmonella species examined is in the range of 50-53% (Hill, 1966). It is noteworthy that the majority of species currently organised in particular genera have DNA base compositions that are quite similar; for example members of the genus Escherichia and the genus Salmonella.

At present, following the suggestion of the Executive Commission of the International Subcommittee on Enterobacteriaceae (1963) the group Proteus-Providencia is sub-divided into four separate sub-groups:

Group Proteus-Providencia

Sub-group

Proteus	P. vulgaris	)	
		)	P. hauseri
	P. mirabilis	)	
Morganella	P. morganii		
Retgerella	P. rettgerii		
Providencia	Providencia	)	P. stuartii
		)	
		)	P. inconstans

The aims of the present studies with regard to various Proteus/Providencia species were:

1) To look at the form and structure of their flagella in order to compare them with those reported for other bacteria; for example with regard to dimensions, "beaded" or "lined" appearance and arrangement of the sub-units.

2) To reinvestigate the molecular weight of Proteus flagellin since there was a discrepancy between the molecular weight reported for P. vulgaris flagellin (Erlander, Koffler and Foster, 1960) and that for other bacterial flagellins.

3) To compare certain aspects of Proteus/Providencia flagellins; for example electrophoretic mobilities, amino acid compositions, peptide maps and amino acid sequences of peptides: firstly with regard to relatedness within the genus (for example P. morganii possesses a guanine plus cytosine content of about 52% whereas other Proteus species possess a guanine plus cytosine content of about 40%); secondly with regard to variant and non-variant amino acid sequences which may indicate regions concerned with the functioning of flagella and the aggregation of sub-units to form the flagellum.

MATERIALS AND METHODS

A. Materials

1. Bacterial cultures

The *Proteus* strains used were obtained from several sources. Those obtained from the National Collection of Type Cultures (NCTC) are designated as such; cultures received as a gift from Professor J. N. Coetzee, University of Pretoria, Pretoria, South Africa, are preceded by the letter C.

2. Chemicals

All reagents used were of analytical grade unless otherwise stated. The sources of the commercially purified proteins used in this investigation were:

Bovine serum albumin	from bovine pancreas - Calbiochem. Crystallised, A grade
Ovalbumin	egg - Sigma. Crystallised and lyophilised, Grade V
Cytochrome c	Horse heart - Sigma. Crystallised, Type 3
Chymotrypsinogen A	Sigma. 6X crystallised
Trypsin	Worthington Biochemical Corp. Crystallised, lyophilised
Chymotrypsinogen	Worthington Biochemical Corp. 3X crystallised
Pepsin	Worthington Biochemical Corp. 2X crystallised
Carboxypeptidase A	Sigma. Dialysed and recrystallised, diisopropylphosphofluoridate (DFP) treated
Carboxypeptidase B	from hog pancreas - Sigma. Frozen solution in 0.1M NaCl, DFP treated

B. Methods

1. Stock cultures

Stock cultures of the organisms used were maintained on the surface of nutrient broth agar (NBA) in 2 x 4 cm screw-capped Bijou bottles. This medium, consisting of 2.5% Oxoid No. 2 Nutrient Broth and 1.2% Oxoid No. 3 agar, was autoclaved at 15 lbs./sq.inch steam pressure for fifteen minutes. The cultures were stored in the dark at 24° and sub-cultured every six months.

2. Microscopy

Preparation of washed bacterial cells

Bacterial cells were washed prior to staining, otherwise background stain was excessive. The cells were taken with a platinum loop from plates on which they had recently swarmed. The loop was gently placed in a test tube containing 2-3 ml of distilled water and left for 10 to 15 minutes. Then, with a gentle rolling action, the bacterial cells were suspended in the water.

a) Flagella staining for light microscopy

The methods of Leifson (1960) and Löffler (1899), both of which cause precipitation of stain on the bacterial flagella, were tried. Leifson's method, even when used for a whole range of carefully controlled staining times, was found to be less successful than that of Löffler:

Löffler's flagella stain

10% alcoholic solution of basic fuchsin	20 ml
3% aniline water	80 ml

Löffler's flagella mordant

20% aqueous tannic acid	100 ml
10% alcoholic solution of basic fuchsin	10 ml
ferrous sulphate	20 g
distilled water	40 ml

Bacterial smears were prepared on glass slides boiled in "Decon" 75 concentrate (2% v/v; Medical Pharmaceutical Developments Ltd.), washed several times in distilled water and flamed before use. Such slides enabled a drop of the bacterial suspension to spread evenly over the surface. The slide was allowed to dry at room temperature to give a thin smear of bacterial cells.

Slides were stained on a staining rack over a sink. Flagella mordant was pipetted over the slide, left five minutes and washed off with tap water. Flagella stain was applied, left three minutes, washed off under tap water and the slide allowed to dry.

Slides were examined under oil immersion using a Zeiss photo-microscope.

b) Preparation of specimens for electron microscopy

Specimens were negatively stained on formvar coated grids. The stains used were:

Uranyl acetate	1%, pH 4.4 (unbuffered)
Phosphotungstate	1%, pH 7.4 (adjusted with N KOH)

Some preparations were fixed in 0.2% formalin (neutralised with 0.1N NaOH) before use. A drop of the preparation to be examined was placed on the grid and excess removed with filter paper. This was followed by stain applied in a similar manner and removed as before. Occasionally, since it was suspected that cells were being washed off the grid, a drop of bovine serum albumin (0.1%) was applied to the grid and a drop of stain mixed with this. Staining times were from 1-4 seconds.

Specimens were shadow cast with 60% gold, 40% palladium alloy at an angle of about 20°.

Specimens were examined in an AEI-6B electron microscope at 60 KV.

3. Growth of bacteria

Highly motile organisms were selected by their ability to swarm from a central inoculum over nutrient agar. Usually 1.2% NBA was used for selecting motile organisms of Proteus vulgaris and P. mirabilis species while motile P. morganii and P. rettgerii cells were selected on 1% NBA.

Bacteria were grown on enriched agar of the following composition:

Tryptone	0.83%
Casein hydrolysate	0.42%
Yeast extract	0.42%
Sodium glycerophosphate	0.42%
Glycerol	0.83%
Sodium lactate	0.42%
Oxoid agar No. 3	1.00%

the constituents being dissolved in distilled water. 750 ml of this medium were dispensed into each of eight one-litre flasks and autoclaved at 15 lbs./sq.inch steam pressure for 15 minutes. The enriched agar, when sufficiently cool, was poured into lidded sterile enamel trays, 25 x 35 x 5 cm, and allowed to set.

The selected motile cells were grown overnight in nutrient broth (Oxoid, No. 2, 2.5%) at 24°. After the cultures had been assessed for motility with a light microscope, about 5 ml of the culture was spread with a sterile glass spreader over the surface of each tray of enriched agar.

The trays were incubated for 2-3 days at 24°.

To harvest the bacteria about 25 ml of water was added to each of the trays and the growth scraped from the surface of the agar with a glass spreader. The suspension, total volume about 250 ml from eight trays, was blended in an MSE blender to remove the flagella from the

bacterial body (Stocker and Campbell, 1959). The flagella were separated from the cells by centrifugation in a Measuring and Scientific Equipment (MSE) 18 high speed centrifuge at 10,000g(9,000 r.p.m.) for forty minutes. All centrifugations were routinely carried out at 10°.

4. Preparation of flagella

The supernatant, containing the suspended flagella, was centrifuged at 35,000g (17,500 r.p.m.) for 1.5 hours in the MSE 18 centrifuge. The flagella pellet, which appeared as a gelatinous clear halo with a darker centre bottom, was soaked up overnight in distilled water at 4°. Sometimes, after the soaking, a magnetic stirrer was used to totally re-suspend the flagella. Continued differential centrifugation was occasionally used to further purify the flagella. When small amounts of purified flagella protein were needed, for example for comparative starch gel studies, sufficient could be obtained by carefully removing the outer portion or flagella halo of a soaked flagella pellet with a spatula and suspending this in distilled water. This method had the advantage that purified flagella could be prepared from up to eight organisms at any one time.

5. Preparation of flagellin

Flagella suspensions were taken to pH 3 by the dropwise addition of N HCl, whilst stirring vigorously. Great care was taken not to allow the pH to drop below 3, otherwise additional bands appeared on subsequent starch gel electrophoresis, possibly as a result of partial deamidation of the protein. P. vulgaris NCTC 4175 flagella were not taken below pH 4.5 since below pH 4 the protein precipitated irreversibly.

The remaining material in suspension in the resultant flagellin solution was removed by centrifugation at 35,000g for 1.5 hours. The clear supernatant obtained was neutralised by the dropwise addition of

N NaOH and brought to 70% saturation with respect to $(NH_4)_2SO_4$. Centrifugation at 10,000g sedimented the flocculant white precipitate. This precipitate was dissolved in a minimal amount of distilled water to give an opalescent pale yellow solution. The solution was dialysed for 16-24 hours against at least six changes of distilled water at 4°. The purified flagellar protein solution was stored at -10°.

6. Reaggregation of flagellin

When purified flagellin solutions were dialysed to remove $(NH_4)_2SO_4$ they usually became very viscous and opalescent. Such solutions were examined in the electron microscope for reaggregated material. This reaggregation was occasionally used as an aid to further purification. The dialysed protein solution was centrifuged at 20,000g for one hour and the pellet obtained redissociated to give purified flagellin.

Flagellin solutions were also polymerised using the method of Wakabayashi, Hotani and Asakura (1969). Purified flagellar protein was dissociated to the monomer by heating in 0.15M NaCl at 60° for 15 minutes. The flagellin solution was dialysed against 0.8M Na_2SO_4 in 10mM phosphate buffer, pH 6.5, for 12 hours at 24°.

7. Characterisation of flagellin

a) Dry weights

Yields of flagellin were estimated by drying flagellin solutions in an oven at 60° to constant values. These yields were related to the total dry weight of bacteria obtained per crop by dry weighting, in a similar way, an aliquot of the suspension obtained after the harvested cells were blended to remove the flagella.

b) Light absorption properties

(i) The absorption spectrum

The absorption spectrum of P. vulgaris NCTC 100 20 flagellin was

measured in 0.1N acetic acid (pH 2.5) and 0.1N NaOH (pH 13). The solutions (1 mg/ml in a 1 cm cuvette) were measured in a Beckman DB-G Grating Spectrophotometer, with an attached recording unit.

(ii) Extinction coefficients

The absorbance of known concentrations of flagellin was measured in distilled water at 215 nm and a curve of absorbance against concentration constructed. This curve was used to obtain an estimate of the extinction coefficient for the flagellin concerned.

(c) Chemical tests on the purity of the protein

Carbohydrate was estimated by the method of Devor (1950).

Phosphate (as inorganic phosphate) was estimated by the method of Fiske and Subarrow (1925).

(d) Starch gel electrophoresis

This method provided a check on the purity of flagellin preparations, the mobility of up to five flagellins being compared on one gel.

The discontinuous method of Poulick (1957) was used. The starch, (Starch hydrolysed; Connaught Medical Research Laboratories, Canada) was made up at 14% in Tris citrate buffer (0.076M tris, 0.005M citric acid; pH 8.65) to which urea had been added to give a concentration of 2 or 3 molar. While swirling, the mixture was gently heated until the starch dissolved, allowed to boil under reduced pressure and poured into perspex formers 11.5 x 8.0 x 0.6 cm. The gels were left at 4° to set. When cool the top layer was sliced off the gel. Flagellar protein, when applied to gels not containing urea, was dissociated to the monomer by heating in 0.05M NH_4HCO_3 at 95° for 30 seconds; when gels contained urea the samples did not need prior dissociation. The samples were applied to 1 cm filter paper strips set in the gel at 2 cm from the cathode end.

The reservoir buffer was 0.3M boric acid; 0.05M NaOH. The gels were run at 350v (about 12 mA per tray) for 4-6 hours. After cooling the gels were horizontally sliced in two and stained for 5-10 minutes in 3% Amido Black in methanol: acetic acid: water (50: 5: 50 v/v). Destaining took place over two days. The wash used was methanol: acetic acid: water, in the same proportions as above.

(e) Polyacrylamide gel electrophoresis

A Shandon Analytical Polyacrylamide Gel Electrophoresis Apparatus (1SAE2734) was used for this procedure. The small pore gels used were:

pH	Buffer	Urea	% acrylamide
8.5	Tris HCl	+/- 2-3M	7.5
2.3	KOH Acetic	+/- 2-3M	7.5

The composition of the buffers was:

pH 8.5	Tris	36.3g
	0.1N HCl	48.0 ml
	"TEMED"	0.46 ml
	H_2O	to 100 ml
pH 2.3	N KOH	48.0 ml
	Glacial acetic acid	2.87 ml
	"TEMED"	0.46 ml
	H_2O	to 100 ml

The gels were made as in the Shandon manual. N,N,N^1,N^1-Tetra methyl-ethylene diamine ("TEMED") (Eastman Kodak) was used as an initiating agent for polymerisation.

Samples were applied in glycerol to the surface of the gel; no spacer gels were used. 30-150 µg of protein was applied per gel,

depending on the protein or mixture in question. When pH 8.5 gels were run Bromophenol Blue was added to the sample as a visible marker indicating the progress of the run.

A Shandon Vokam constant voltage/constant current D.C. power supply was adjusted to deliver 5 ma to each running tube (voltage about 100 v). pH 8.5 gels were generally run for one hour, pH 2.3 gels for a little longer. The gels were removed from their running tubes with a water filled syringe. The dye fronts, if present, were marked by fine steel wire. Gels were stained in either Coomassie Blue (0.25%(w/v)in 454 ml of methanol to 46 ml of acetic acid, filtered before use) or in 1% (w/v) nigrosin in 7%(v/v) acetic acid. The gels were destained for several days in 7% (v/v) acetic acid.

8. Molecular weight studies

Both gel filtration and polyacrylamide gel electrophoresis have been used to determine the molecular weights of Proteus flagellins. Gel filtration was initially used but for reasons to be explained the final method of choice employed measurement of the migration of flagellins and marker proteins on electrophoresis in acrylamide gels containing sodium dodecyl sulphate (SDS).

a) Gel filtration

Extreme care was necessary to ensure evenly poured columns for this work. 100 cm Pharmacia columns with sample applicators were used; Sephadex G100, swollen in accordance with the manufacturers instructions, was the gel filtration medium. Columns were poured in the desired buffer and allowed to equilibrate for at least 24 hours before they were used. The columns were repoured every week. The buffer most commonly used was 0.05M Tris HCl, pH 7.5, 0.1M in KCl (Andrews, 1964). Guanidine hydrochloride at a concentration of 1M, and urea at concentrations from 0.5-2M were also used.

The marker proteins and the amounts placed on the column were:

Protein	Amount on column
Bovine serum albumin	0.32 mg
Ovalbumin	0.24 mg
Chymotrypsinogen	0.24 mg
Cytochrome c	0.24 mg

Flagellar protein was dissociated before use by heating in 0.1M NH_4HCO_3 or by adding urea to a concentration of 2M. Approximately 0.3 mg of flagellin was applied to a column when the behaviour of this protein was examined.

The marker proteins and sample flagellins, either singly or in various combinations, were layered (in 5% sucrose) on the top of the column. The sample volume was usually 1.05 ml. The effluent, at a flow rate of 30 ml per hour, was collected in 2.5 ml fractions using the drop counting mechanism of an LKB 7000 Ultrorac fraction collector.

The proteins were assayed by reading the absorbance of each fraction at 215 nm in the spectrophotometer. An additional reading was made for cytochrome c which specifically absorbs at 412 nm.

b) Polyacrylamide gel electrophoresis

Following the technique of Weber and Osborn (1969) polyacrylamide gel electrophoresis was used to determine the molecular weights of proteins. Gels were made up in the following buffer systems:

Buffer		% acrylamide	% SDS	Reference
pH 8.5	Tris HCl	7.5	0.1	Shandon manual
pH 8.5	0.05M phosphate	10.0	0.1	Weber and Osborn (1969)

The gels were made and run as detailed earlier. Sample proteins and

flagellins were made up in SDS buffer and incubated at 37° for one hour before use. Mixtures of the proteins and Bromophenol Blue were applied in glycerol to the tops of the gels. Tris HCl gels were run for 1.25 hours; phosphate gels for 2-3 hours, both at 5 ma per tube (voltage = about 100 v).

When the run was complete the gels were removed and the dye fronts marked with fine steel wire. The gels were fixed for 2 hours in 20% (w/v) sulphosalycilic acid, rinsed in 7% (v/v) acetic acid and stained for one hour in Coomassie Blue in methanol/acetic. The fixation in sulphosalycilic acid removes much of the SDS and lessens the precipitate formed by interaction of the Coomassie Blue with the anionic detergent. The extent of migration of the proteins and of the Bromophenol Blue was measured against centimetre graph paper overlain by a glass sheet. The results were expressed as the distance of migration of the protein (DPM) relative to the migration of the dye marker (DDM) - (DPM/DDM).

9. Cleavage of flagellin

a) Acid hydrolysis

(i) Total acid hydrolysis

Pyrex hydrolysis tubes, 15 x 0.75 cm with a partly drawn out neck, were used for total acid hydrolysis. The sample of 1-6 mg of protein in 6 N HCl was placed in the tube, the tube evacuated with a water pump, and the neck drawn out and sealed in a flame. The samples were heated at 110° and removed at periods from 16-72 hours. Acid was removed *in vacuo* over P_2O_5 and NaOH pellets; the last traces of acid were removed by wetting and redrying *in vacuo*.

(ii) Partial acid hydrolysis

Whole protein was exposed to 6 N HCl for 3 days at 37° in covered test tubes (Naughton et al, 1960 - see Snell and Offord, 1972). The cores

resulting from the enzymic (tryptic) digestion of whole protein were more resistant and were partially hydrolysed in 12 N HCl at 100° for 30 minutes. Acid was removed as above.

b) Enzymic hydrolysis

(i) Tryptic and chymotryptic digestion

The flagellar protein sample was brought to 0.05M with respect to NH_4HCO_3 to give a final concentration of about 10 mg of flagellin in 1 ml of solution. The solution was heated in a water bath at 95° for 30 seconds and rapidly cooled under running tap water. Trypsin or chymotrypsin was added at an enzyme to substrate ratio of 1 : 50 (w/w). Digestion was allowed to proceed for 1.5 - 2 hours. The resultant viscous digests were frozen and the "core" material that precipitated out centrifuged down at 6,000g using an MSE bench centrifuge.

(ii) Peptic digestion

The pH of the flagella protein solution (about 10 mg in 1 ml) was lowered to pH 2 with 0.1 N HCl. Pepsin was added in the ratio of 1 : 50 (w/w) of enzyme to substrate and the digestion allowed to proceed for 1 hour at 37°. The digest was either used directly or stored at -10°.

(iii) Carboxypeptidase A and B digestion

Carboxypeptidase A was prepared immediately before use by washing the crystals by centrifugation to remove contaminating amino acids and then suspending these in 0.1% (w/v) sodium bicarbonate, cooled in an ice bath. 0.1 N NaOH was added dropwise with thorough mixing until the protein dissolved. The pH was brought back to 8-9 by the addition of 0.1 N HCl.

The carboxypeptidases used (A and B) were treated with diisopropyl-phosphofluoridate (DFP). This inhibits the activity of other pancreatic proteases, especially chymotrypsin.

Digestion of flagellin with carboxypeptidases was carried out in 0.2M N-ethylmorpholine acetate buffer, pH 7.5. The sample, volume 1 ml, containing about 5 mg of flagella protein, was heated at 90° for 30 seconds and rapidly cooled. Carboxypeptidases A or B were added in the ratio of approximately 1 : 40 (w/w) of enzyme to substrate and the reaction allowed to proceed for between 0 and 120 minutes. To terminate the reaction 1.5 volumes of acetone were added and the precipitate removed by centrifugation. The supernatant was evaporated and dissolved either in 0.1 ml of water for qualitative analysis on paper or 5 ml of pH 2.2 citrate buffer for quantitative amino acid analysis.

c) Cyanogen bromide cleavage of flagellin

Cyanogen bromide specifically and almost quantitatively splits peptide chains on the carboxyl side of the methionine residues, converting methionine into homoserine lactone. Hence a protein with three methionine residues should yield four fragments on reaction with cyanogen bromide. These large fragments are extremely useful for sequence studies since they provide long sections of the amino acid sequence in a manageable form.

Cyanogen bromide was reacted with flagellin under the following acidic conditions:

70% (v/v) formic acid	(Steers, Craven and Bethune, 1965)
0.1 N HCl/6M urea	(Gross and Witkop, 1962)
0.2 N HCl/6M urea	(Gross and Witkop, 1962)

The reaction was carried out in a Thunberg tube. A 50-fold excess (w/w) of cyanogen bromide was added to a flagellar protein solution of about 20 mg in 1 ml and the reaction allowed to proceed at 24°. After 3 days at 24° the reaction mixture was either freeze dried or briefly dialysed against several changes of distilled water to remove excess reagents. Freeze dried samples were solubilised in 0.2 N acetic acid or

citraconylated with citraconic anhydride.

The products of the reaction were examined initially by polyacrylamide gel electrophoresis in pH 2.3, 6M urea gels or at pH 8.5 in gels containing 0.1% SDS. Chromatography of the solubilised digests was performed on Sephadex G75 columns (100 x 2.5 cm), run in 0.2N acetic acid. Column flow rates were about 25 ml per hour; 2.5 ml fractions were collected in an LKB Ultrorac fraction collector, using the drop counting mechanism. 0.2 ml aliquots of the fractions were assayed using the method of Moore and Stein (1954). Fractions were pooled as appropriate and further examined by polyacrylamide gel electrophoresis.

10. Amino acid analysis

a) Qualitative identification of amino acids by paper electrophoresis and chromatography

The amino acids of peptide hydrolysates were usually identified by a combination of paper electrophoresis and chromatography. The sample was spotted near one corner of the paper; Whatman, Grade 2 Chroma; 20 x 40 cm (Fig. 1). The buffer used for electrophoresis was acetic acid : formic acid : water (15 : 5 : 80 (v/v), pH 1.9. Using a pipette the paper was wetted carefully with this buffer and subjected to electrophoresis in a flat bed high voltage electrophoresis apparatus (Camag), at 3,000 volts for 25 minutes. After air drying the paper was rolled into a cylinder and subjected to ascending chromatography in a Shandon Unikit tank. The running solvent was the upper phase of a mixture of n butanol : acetic acid : water (4 : 1 : 5 v/v). The paper was removed, air dried and dipped in ninhydrin solution (0.2% ninhydrin (v/v) in acetone). Figure 1 shows the separation by this system of a mixture of standard amino acids.

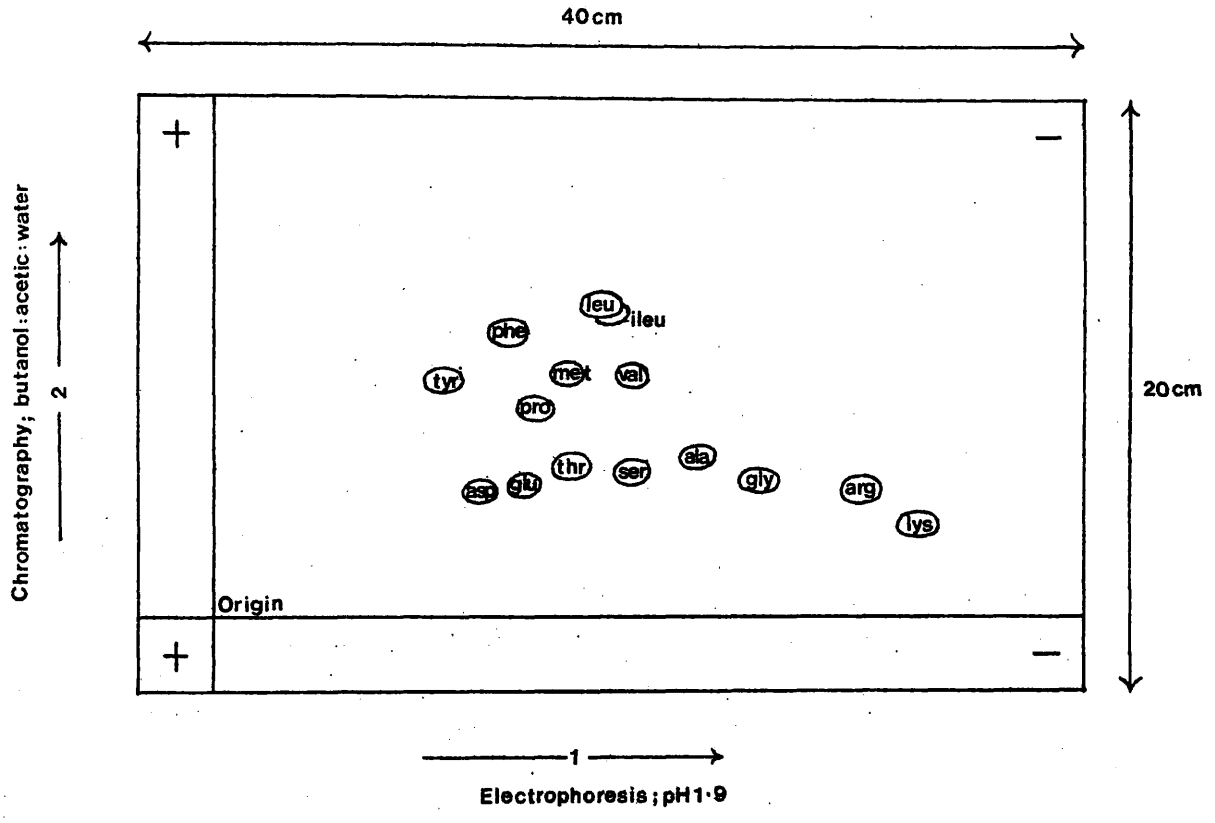


Fig.1. Amino acid map showing the separation of a standard mixture of amino acids.

b) Quantitative amino acid analysis

Quantitative automated analysis of amino acids

Dried down hydrolysates were dissolved in 5 ml of pH 2.2 citrate buffer. 2 ml of each hydrolysate were applied to the 150 and 15 cm columns of an automatic amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex, England), such that from 0.5 to 2.0 mg were analysed according to the method of Spackman, Stein and Moore (1958).

11. Maleylation and citraconylation of protein and peptides

a) Maleylation

Flagella protein at a concentration of about 15 mg/ml was treated with a final concentration of 50 mM maleic anhydride at pH 9 (Butler, Harris, Hartley and Leberman, 1967). The pH was maintained by the addition of 2 N NaOH. The protein after completion of the reaction, which took about 5 minutes, was desalted by dialysis against several changes of water at 4°.

b) Citraconylation

Flagellar protein at 15 mg/ml was treated with a final concentration of 150 mM citraconic anhydride (Dixon and Perham, 1968). The pH was maintained at 8 by the addition of 3 N NaOH. The reaction was complete within about 5 minutes and the protein was desalted as above.

Unblocking of maleylated or citraconylated proteins or peptides

Unblocking of the lysine residues blocked by maleylation or citraconylation takes place at low pH. Maleylated and citraconylated proteins were suspended overnight in pH 3.5 electrophoresis buffer (pyridine : acetic acid : water; 1 : 10 : 100 v/v) and then digested further or examined electrophoretically.

12. Peptide analysis

a) Peptide analysis on paper

(i) Peptide maps

Insoluble "core material" was removed from tryptic and chymotryptic digests by centrifugation. The "core material" sedimented more easily if the digest previously had been frozen. Peptic digests did not contain any appreciable amount of "core material".

Digests were spotted onto Whatman 3 mm chromatography paper, drawn out as in Figure 2. Chromatography was performed in a descending manner for 16-17 hours in a covered Panglass Shandon 500 Chromatank at 24°. Up to three papers were placed in the tank at one time. The solvent used for chromatography was the upper phase of a mixture of n butanol : acetic acid : water (4 : 1 : 5 v/v). The papers were thoroughly air dried and subjected to electrophoresis in a flat bed electrophoresis apparatus (Camag) at 2,000 volts for one hour. When dry the paper was dipped in 0.2% ninhydrin in acetone, dried and heated over a hot plate until spots just began to appear. Peptides which gave an initial strong yellow colour (indicating N terminal glycine or amide residues) were marked at this stage. The chromatogram was then left to develop overnight, away from direct light.

(ii) Specific reagents for the identification of peptide amino acids

The methods used were those of Easley (1965) with some slight modification.

The Pauly reaction was used for the identification of histidine. The reagents used were:

Solution A: 4.5g sulphanilic acid + 5 ml of concentrated HCl, made to 500 ml with water. Warmed slightly to dissolve.

Solution B: 5% (w/v) $NaNO_2$ (aqueous); freshly prepared.

Solution C: 10% (w/v) Na_2CO_3 (aqueous).

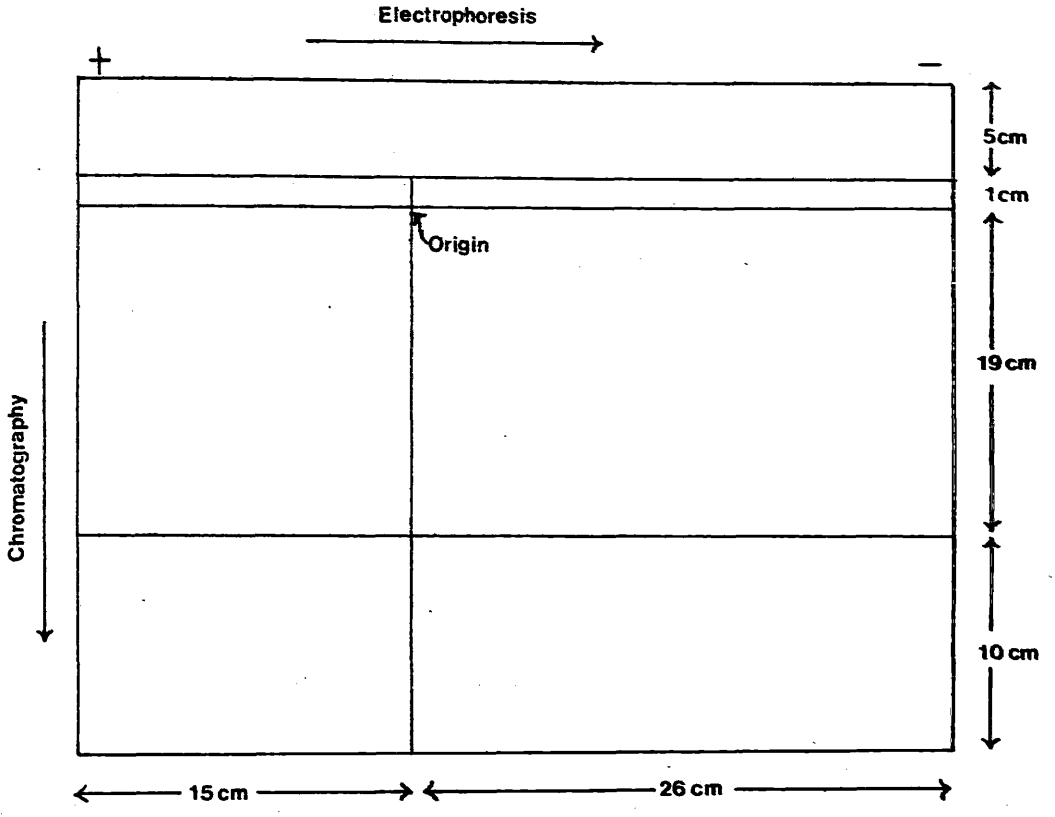


Fig.2 Diagram to show the dimensions of the paper used for a peptide map.

Solutions A, B and C were chilled separately in an ice bath. One part B was added to two parts of A and the paper lightly sprayed with this mixture. Then the paper was sprayed very lightly with C until spots appeared without fading. Histidine spots were orange pink, tyrosine spots tended towards brown. After drying the paper was dipped in ninhydrin to localise any histidine or tyrosine spots detected.

Tyrosine containing peptides were identified by dipping the chromatogram, previously dipped in ninhydrin in the following reagents:

Solution A: 0.1% (w/v)α nitrosonaphthol in acetone.

Solution B: 10 ml concentrated HNO_3 + 90 ml of acetone (freshly prepared).

The paper, after dipping in A, was thoroughly dried before being dipped in B. After 5-10 minutes drying the chromatogram was warmed carefully over a hot plate. Tyrosine spots were rose coloured, the background light yellow.

Arginine peptides were identified by the Sakaguchi reaction. The solutions used were as follows:

Solution A: 0.2% (w/v) hydroxyquinoline in acetone

Solution B: 0.5 N NaOH (20 ml) + 0.3 ml bromine

The chromatogram was dipped in A, allowed to dry and sprayed very gently with B. Great care had to be taken not to overspray. Arginine spots were orange pink and transient. After drying the paper was dipped in ninhydrin to locate the arginine peptides detected.

(iii) Identification of the C terminal peptide

Equal quantities of flagellar protein (5-10 mg in about 1 ml) were taken in glass centrifuge tubes (8 x 1.5 cm). The protein was precipitated by the addition of 1.5 volumes of acetone, centrifuged down at 6,000g in an MSE bench centrifuge and the supernatant, containing free amino acids, if present, removed. 1 ml of water and 1 ml of 0.4M

N-ethylmorpholine acetate at pH 7.5 was added to the precipitate.

The suspensions were heated at 95° for 30 seconds to solubilise the protein. 0.01 ml of carboxypeptidase B - DFP containing 20 µg of enzyme was added to one of the tubes. Both were incubated at 37° for one hour. 1.5 volumes of acetone (3 ml) was added to each tube to precipitate the flagellin. After centrifugation at 6,000g the supernatants were removed and the amino acids present in these identified by paper electrophoresis and chromatography.

The flagellin precipitates were suspended in 0.25M NH_4HCO_3 and heated at 100° for one minute to solubilise the protein. After cooling, 0.05 mg of trypsin was added to each tube. Both tubes were incubated at 37° for 2 hours and the digests, after freezing, thawing and removal of the "core" material, mapped by chromatography and electrophoresis.

(iv) Peptide purification on paper

Peptides were routinely purified by electrophoresis and/or chromatography. Electrophoresis was at one of the following pH's:

pH 6.5 Pyridine : acetic acid : water (10 : 1 : 100 v/v)

pH 3.5 Pyridine : acetic acid : water (1 : 10 : 100 v/v)

pH 1.9 Acetic acid : formic acid : water (15 : 5 : 80 v/v)

The sample was applied as a thin band 6 cm from the anodic end of the paper (Whatman 3 mm) for pH 1.9 and pH 3.5 electrophoresis and at 15 cm for pH 6.5 electrophoresis. Marker strips were developed with ninhydrin and the relevant zones cut out and eluted by immersion in water. Peptides were occasionally eluted from the paper by suspending the relevant portion between two glass rods held together with rubber bands and slowly applying distilled water to the top of the paper until the peptide was washed into a beaker placed underneath. About 0.5-1.0 ml was necessary to elute the peptide.

Chromatography in n butanol : acetic acid : water (4 : 1 : 5 v/v) was used to separate those peptides that could not be separated electrophoretically. The peptides were eluted as above.

b) Ion exchange chromatography

The method of Schroeder (1967) was used for separation of peptides on cation exchange resins. A column (1 x 100 cm) was packed with prepared Dowex 50-X2 resin. The column was equilibrated with prepared pH 3.1 buffer and the sample (volume approximately 2 ml, adjusted to pH 3.1) applied to the column. The buffers used were:

0.2M pyridine acetate, pH 3.1	333 ml
2.0M pyridine acetate, pH 5.0	666 ml

An elution gradient of increasing concentration and pH was maintained by pumping the pH 5.0 buffer at a constant rate into a mixing chamber containing the pH 3.1 buffer. This mixture, of increasing molarity and ionic strength was pumped from the mixing chamber through the column at a similar rate to the input into the mixing chamber.

Fractions were collected in an LKB Ultrorac fraction collector, using the drop counting mechanism. The fraction size was usually in the region of 1.5 ml.

Assay of column effluent

The method used was that of Moore and Stein (1954). Aliquots of 0.1-0.3 ml were taken from each fraction into a fresh test tube. 1 ml of 2.5 N NaOH was added and the tubes heated in a covered boiling water bath for 1.5 hours. After cooling, 1 ml of 30% acetic acid was added to each tube, the contents mixed and 1 ml of ninhydrin reagent added to each.

Ninhydrin reagent	Ninhydrin	2.0 g
	Hydrindantin	0.3 g
	Methoxyethanol	75 ml
	4M sodium acetate	25 ml

The tubes were heated in a boiling water bath for exactly 15 minutes, cooled and 2 ml of 50% industrial methylated spirits added to each. After vigorous shaking the absorbance of the contents of the tubes was read in a spectrophotometer at 570 nm.

13. Sequence analysis

a) Sequential Edman Degradation

Edman's phenyl isothiocyanate reaction was used to sequentially degrade a peptide from the N terminal end. The N terminal amino acid of each degradation product was identified directly with the dansyl chloride technique.

The method used (with some minor modification) was that of Gray (1967). Several peptides were investigated simultaneously. The peptide solution (of which the amino acid composition was already known) was transferred to a small piccolo centrifuge tube (7.5 x 1.0 cm) and dried down in an air stream. The sample was redissolved in 200 µl of 50% pyridine, a suitable sample removed for end group studies, and the main volume restored to 200 µl with 50% pyridine. 10 µl of PTC (5% phenyl isothiocyanate in pyridine, v/v) was added, the tube covered with a double layer of Parafilm and left for one hour at 45°. Excess reagent was removed in a desiccator containing P_2O_5 and NaOH pellets, evaporated with an efficient water pump and kept at 60° in a water bath. Samples required at least 30 minutes for the complete removal of all excess reagents; when dry the contents of the tube were a pinkish brown and crystalline in appearance.

200 µl of anhydrous trifluoracetic acid (TFA) was carefully added to the tube to cleave the phenylthiocarbamyl peptide. The tube was quickly covered with Parafilm and incubated at 37° for 20 minutes. TFA was removed in vacuo at 60°. This took about five minutes. 400 µl of water was added to the residues and diphenylurea (DPTU) extracted using 3 x 1.5 ml of n butyl acetate. The two phases were mixed thoroughly with a vortex mixer and separated by centrifugation. The top layer containing DPTU and probably the phenylthiocarbamyl amino acid was discarded. A suitably sized sample was removed for dansylation and the remainder submitted to another cycle of the degradation procedure.

b) Dansylation

A graduated 1 ml hypodermic syringe with a fine bore extension tube was used to place the sample removed for dansylation in a pyrex hydrolysis tube (15 x 0.75 cm) with a partly drawn out neck. This sample was dried down in an air stream and redissolved in 10-15 µl of $NaHCO_3$ solution (0.2M in deionised water). The sample was redried and redissolved in deionised water. The pH was checked to ensure that it was still above 7.5. An equal volume of dansyl chloride solution (1 - dimethylaminonaphthalene - 5 - sulphonyl-chloride (DNS-Cl); Koch Light Laboratories, Colnbrook, England) at a concentration of 2.5 mg per ml in acetone was added, the tubes covered with Parafilm and incubated at 37° for one hour. During this time the solution became colourless, due to the hydrolysis of excess reagent to the sulfonic acid (DNS OH). The solution was dried down in an air stream.

Hydrolysis

50 µl of 6 N HCl was added to the dried residue, the tubes evacuated and quickly sealed in a batswing flame. The tubes were heated for 4-5 hours at 110°. The tubes were opened and dried down over P_2O_5 and NaOH pellets, in vacuo.

Because of the decreased accessibility of the end group to the reagent dansylation of whole protein rather than of peptides requires much more dansyl chloride. 0.75 ml of strong dansyl chloride solution (20 mg/ml in acetone) was added to 0.75 ml of 0.5M $NaHCO_3$ containing about 5 mg of flagellin. The urea used was of "Aristar" grade (BDH Chemicals); free of both ammonia and cyanate. The mixture was left to react for three hours at 37°. The protein was desalted by three hours dialysis against distilled water. Hydrolysis was as previously described for dansylated peptides.

c) Identification of dansyl amino acids

(i) Electrophoresis

Samples were extracted with water saturated ethyl acetate to remove excess dansyl hydroxyl; dried down and taken up in 50% pyridine (v/v). Samples were spotted with a finely drawn pasteur pipette onto Whatman Grade 2 Chroma paper (20 x 40 cm). The origin was placed close to the anode since no dansyl amino acid has a negative charge. The paper was carefully wetted with pH 1.9 electrophoresis buffer and subjected to electrophoresis at 3,000 volts for thirty minutes. The paper was thoroughly dried and the dansyl amino acids visualised with a long wave ultra violet lamp (maximum emission 365 nm).

(ii) Thin layer chromatography

This was the method of choice when small amounts of dansyl amino acids were to be identified (Woods and Wang, 1967; Weiner, Platt and Weber, 1972). Polyamide sheets (15 x 15 cm), coated on both sides, were obtained from BDH Chemicals Ltd., Poole, Dorset, England. These sheets were cut with scissors to each give nine sheets of 5 x 5 cm. Samples were applied 1 cm from one corner with a finely drawn pasteur pipette. The sample spots were not allowed to exceed 2 mm in diameter. The spots were dried in a hot air stream between each application.

Standards containing known amino acids were spotted on the other side of the plate. The plates were dried for a further 5 minutes in a cold air stream before running.

Solvent 1	Water : formic acid	98.5 : 1.5
Solvent 2	Benzene : acetic acid	9 : 1
Solvent 3	Ethylacetate : methanol : acetic acid	20 : 1 : 1

Solvents were stored in tightly capped bottles at room temperature. Chromatography was performed in 250 ml beakers covered with a stretched sheet of Parafilm. Plates were dried and examined with an ultra violet lamp as above. Figure 3 shows the separation of a standard mixture of known dansylated amino acids by this system.

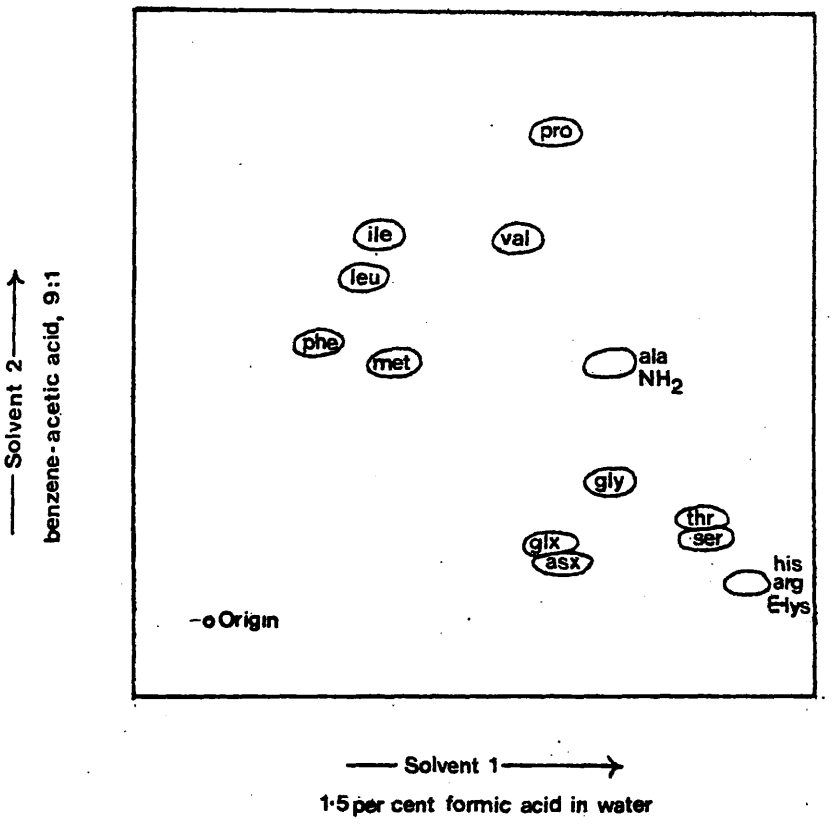


Fig.3 Chromatography of dansylated amino acids on thin layers.

Dansylated amino acids visualised with a long wave ultraviolet lamp.

RESULTS

1. Bacterial growth

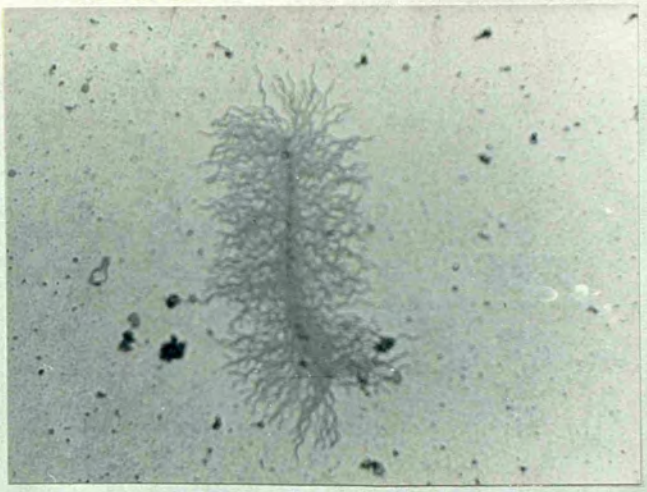
After serial culture *Proteus* strains readily swarmed over the surface of nutrient agar. *P. vulgaris* and *P. mirabilis* strains swarmed on 1.2% nutrient agar; *P. morganii* and *P. rettgerii* strains, however, required softer agars of 0.8 - 1.0%. The periodicity of swarming, characterised by swarming zones in the form of concentric rings, was observed. Actively swarming strains could cover the surface of a moist plate from a central inoculum in 12 hours. Other strains swarmed more slowly and needed up to 48 hours to cover the surface of a plate. The occurence of swarming was dependent on the state of the nutrient agar plates. Usually *Proteus* strains would not swarm unless the plates had been freshly poured.

2. Microscopy of bacterial cells

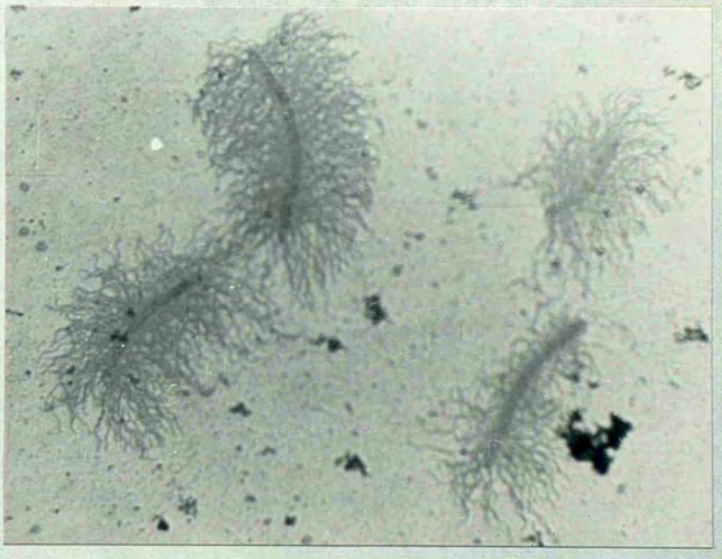
a) Light microscopy

When a smear prepared from a recently swarmed culture was stained and examined, extremely long swarmer cells were noted amongst the ordinary bacterial cells. The swarmer cells of *P. vulgaris* NCTC 100 20 were up to 50 times the normal cell length (Fig. 4). Swarmer cells of *P. mirabilis* NCTC 6197 were 5 to 10 times the normal cell length while *P. morganii* NCTC 232 and *P. rettgerii* NCTC 7475 swarmer cells were only 2 to 3 times the normal cell length (Fig. 5).

The cells of *P. vulgaris* NCTC 100 20 and *P. mirabilis* NCTC 6197 generally possessed the greatest numbers of flagella. 300 to 400 flagella were present on the long swarmer cells of *P. vulgaris* NCTC 100 20; the numbers were proportionately reduced for shorter cells of this strain.

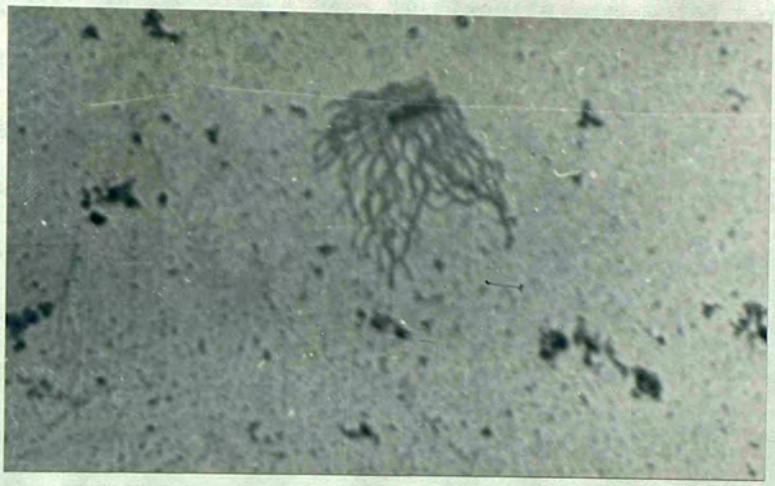


P. vulgaris NCTC 100 20 x 1,250

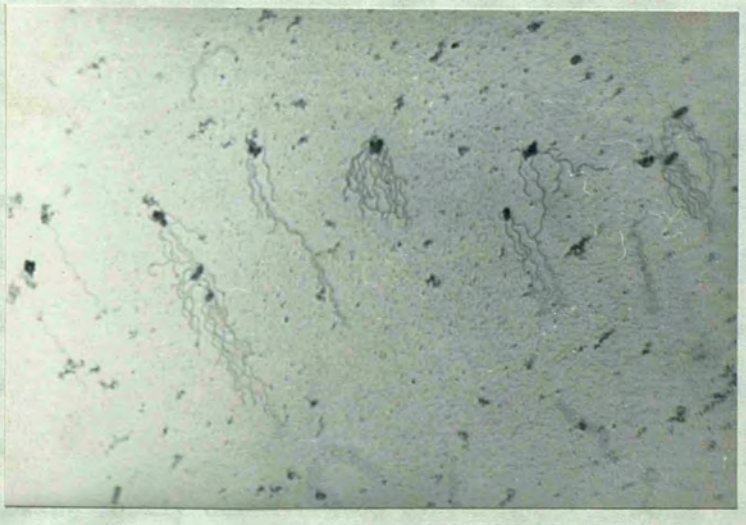


P. vulgaris NCTC 100 20 x 1,250

Fig. 4 Flagellated cells stained by the method of Löffler; examined with a light microscope



P. mirabilis NCTC 6197 x 1,250



P. morganii NCTC 232 x 1,250

Fig. 5 Flagellated cells stained by the method of Löffler; examined with a light microscope

P. rettgerii NCTC 7475 cells usually possessed least flagella. For this organism commonly less than 10 flagella were noted per bacterial cell. Since no long swarmer cells were noted, in contrast to the P. vulgaris and P. mirabilis cultures, this is partly an effect of total cell length. However, P. vulgaris cells of comparable length also had proportionately more flagella.

P. vulgaris NCTC 100 20 cells grown in liquid medium adjusted to pH 5 showed curly flagella, that is with a shorter wavelength than the flagella of cells grown at the usual pH of 7.2. The cells were motile. Amongst the strains studied no aberrant forms, other than those produced by a change in pH of the growth media, were noted.

b) Electron microscopy

Bacterial cells and their attached flagella were easily seen with shadowing and negative staining techniques (Figs. 6, 7, 8.). Cells fixed in formalin showed fragmented flagella; this is attributed to a brittleness as a result of the fixation procedure. Frequently mucilaginous material surrounded the flagella, particularly those of swarmer cells. To remove this material a more vigorous washing technique using low speed centrifugation was employed. However, this resulted in the large scale removal of flagella and was abandoned.

Shadowing easily demonstrated flagella but gave no details of their structure.

1% uranyl acetate was the most successful negative stain. Phosphotungstate did not spread evenly over the surface of the formvar coated grids and stained the cells very heavily even when the staining time was minimal. The benefits obtained from the addition of bovine serum albumin to the stains were debatable. The stains did spread more evenly and fewer cells were washed off the grid but more background staining was noted and it was difficult to obtain sufficient contrast.

Fig. 6 P. vulgaris 100 20 flagella x 80,000; shadowed with gold/palladium alloy

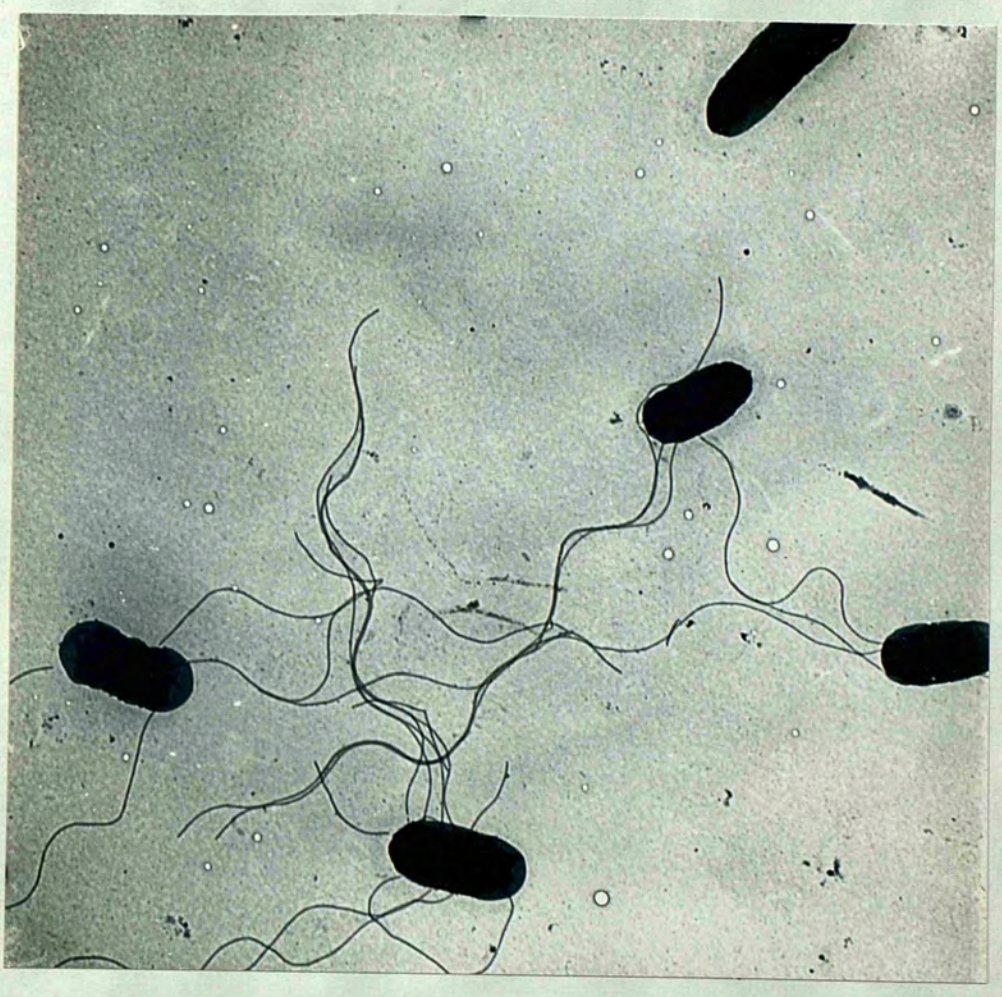


Fig. 7 P. morganii NCTC 232 cells x 15,000;
negatively stained with uranyl acetate

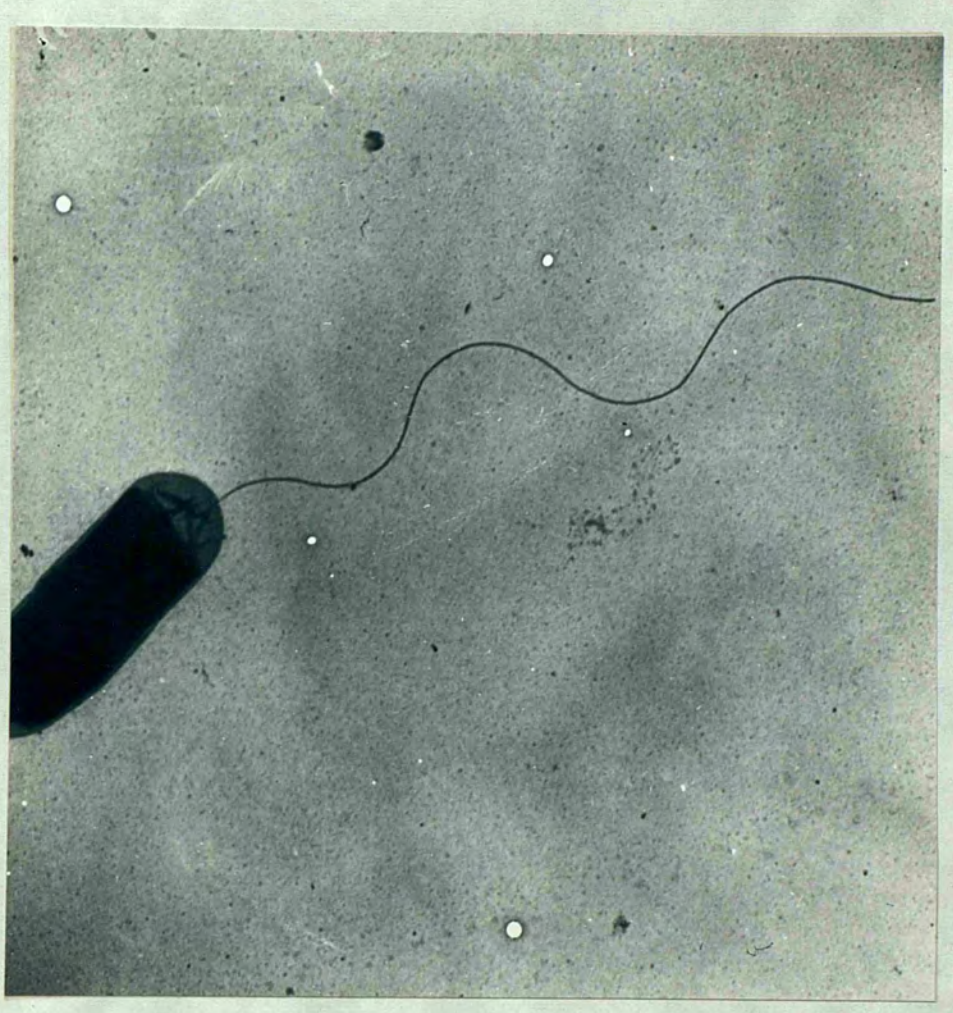


Fig. 8 P. morganii NCTC 232 cell x 30,000;
negatively stained with uranyl acetate

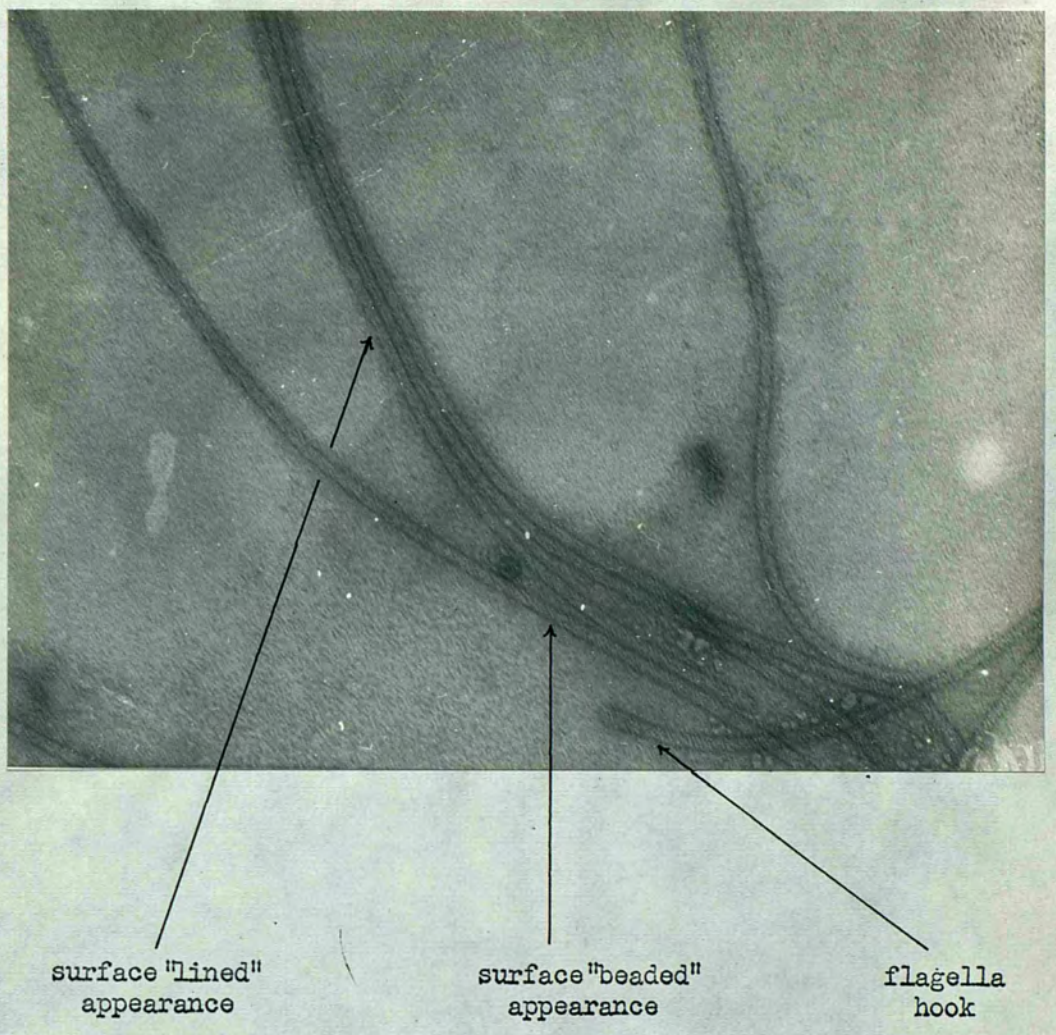


Fig. 9 P. vulgaris NCTC 100 20 flagella x 160,000; negatively stained with uranyl acetate

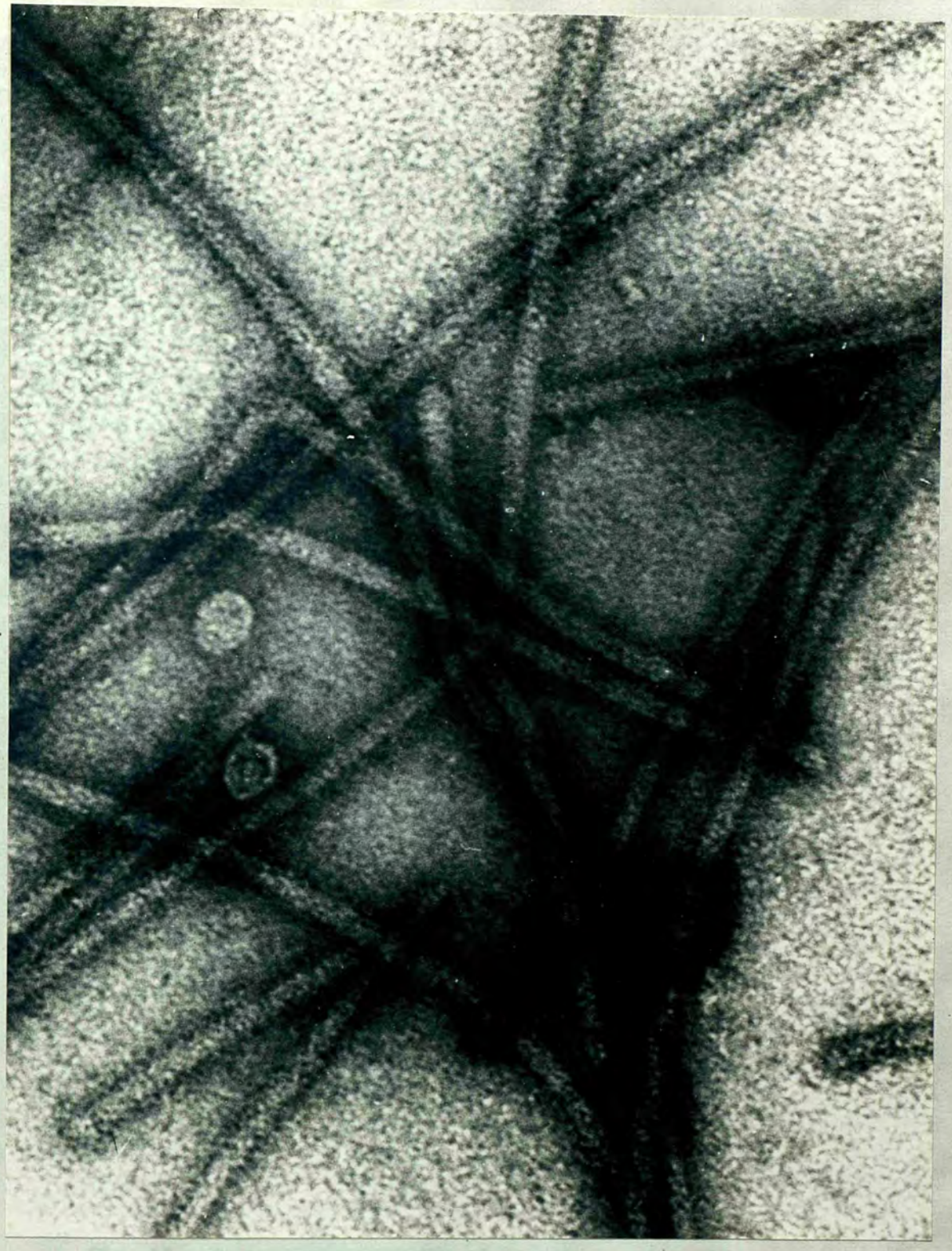


Fig. 10 P. vulgaris NCTC 100 20 flagella x 360,000;
negatively stained with uranyl acetate

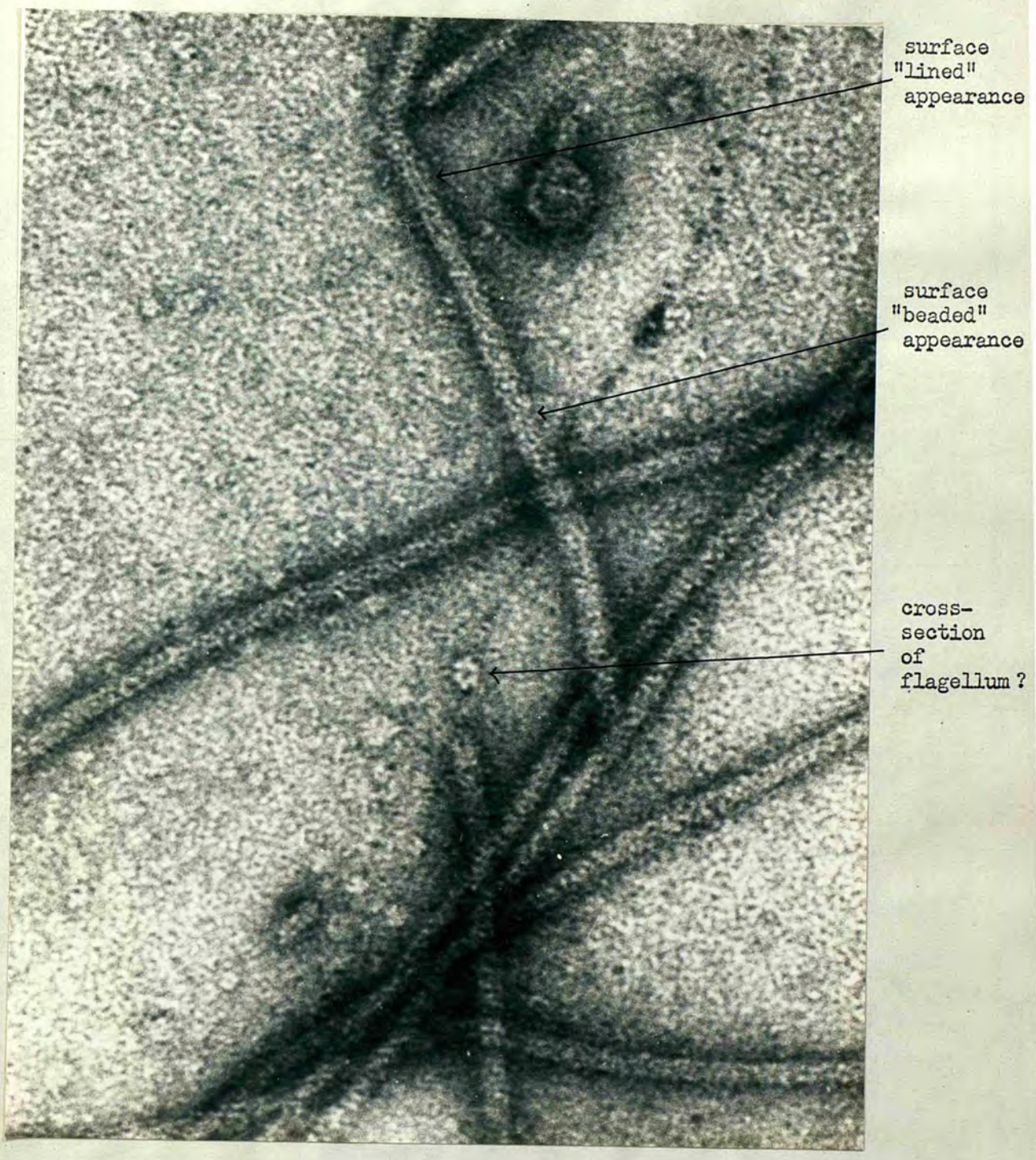


Fig. 11 P. vulgaris NCTC 100 20 flagella x 360,000;
negatively stained with uranyl acetate

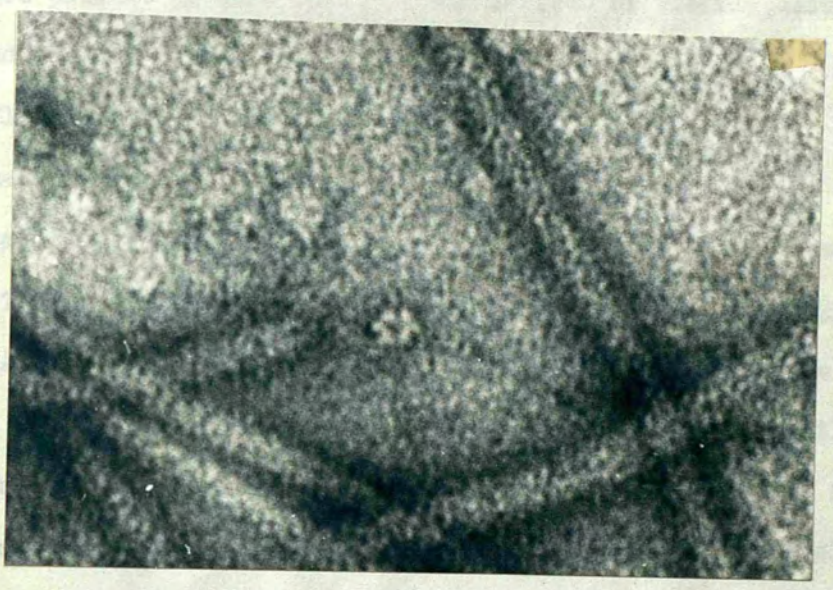


Fig. 12 Possible cross section of flagellum of P. vulgaris NCTC 100 20 x 410,000; negatively stained with uranyl acetate

Detailed examination of the flagella of P. vulgaris NCTC 100 20 revealed both "beaded" and "lined" surface appearances, often on the same flagellum. When the surface of the flagellum appeared "beaded" the surface globular units were arranged in oblique rows; three or four globular units were present in each row. If the surface of the flagellum presented a "lined" appearance two or three lines running in the direction of the flagella axis were evident (Figs. 9, 10, 11). Flagella hooks were sometimes seen (Fig. 9).

Occasionally possible cross sections of flagella were noted (Figs. 11, 12). Six sub-units were observed, arranged in two groups of three. A slight gap was noted between the end sub-units of the triplets. Attempts to cut cross sections of flagella embedded in agar failed since the flagella dissociated during the procedure. Only presumed flagellin sub-units approximately grouped together in the form of a flagellum waveform could be found.

3. Yields of bacteria and bacterial flagellar protein

Between 2-3g dry weight of bacteria were obtained per tray of medium. Flagellar protein yields depended on the species in question, since some species and strains were more heavily flagellated than others. Normally, yields of 12-80 mg of purified flagellar protein per tray were obtained. P. vulgaris NCTC 100 20 yielded at least 500 mg of flagellar protein per crop of eight trays; 100-250 mg of flagellar protein were obtained from crops of P. morganii NCTC 232 and P. rettgerii NCTC 7475. These figures represent, relative to the dry weight of bacteria harvested, flagellar protein yields of 0.4-4.0%.

4. Purity of the flagellins

a) Starch gel and polyacrylamide gel electrophoresis

No great difficulties were encountered in the purification of flagellar protein. The outer portion of a soaked flagella pellet examined

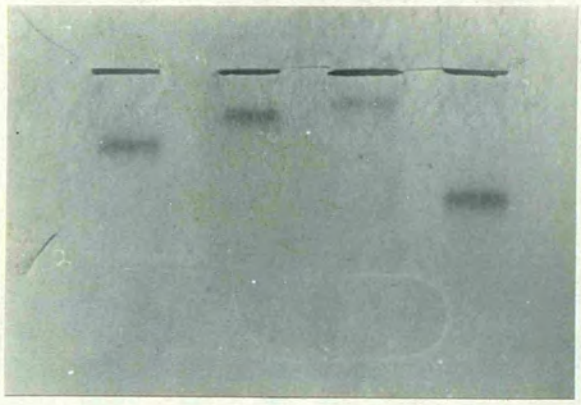
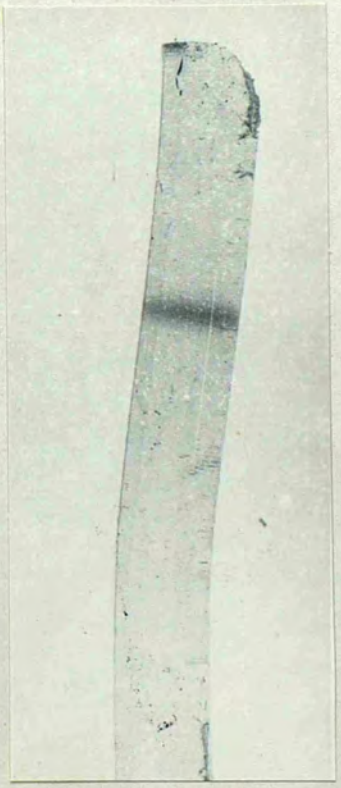


Fig. 13 Flagella proteins purified by acid disaggregation; examined by starch gel electrophoresis

Gel buffer: Tris citrate, pH 8.65, 2M urea



P. vulgaris NCTC 100 20

Fig. 14 Flagella protein (P. vulgaris NCTC 100 20) purified by acid disaggregation; examined by polyacrylamide gel electrophoresis

Gel buffer: Tris HCl, pH 8.5

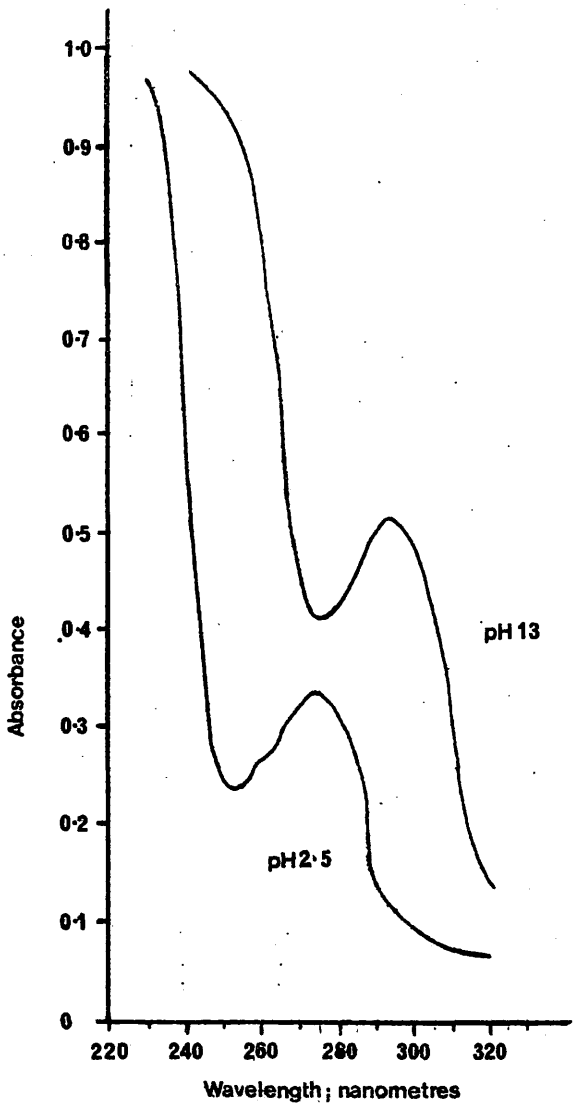


Fig.15 The absorption spectra of P.vulgaris N.C.T.C. 100,20 flagellin in 0·1N Acetic Acid and 0·1 N NaOH. 1mg/ml solution; 1cm cuvette.

by starch gel electrophoresis at pH 8,6 in 2M urea, gave a single protein band corresponding to the flagellin in question. To obtain larger amounts of flagellar protein the complete purification procedure, including pH 3 treatment, was followed. When, during the purification, the pH of a flagella suspension was lowered, the solution became extremely viscous between pH 5 and pH 4; then with the addition of more acid to pH 3 the viscosity decreased rapidly. Flagellins purified in this way examined by starch gel electrophoresis (both in the presence and absence of urea) or by polyacrylamide gel electrophoresis at pH 7.5 (both with and without urea) showed single protein bands (Figs. 13, 14). However, a number of bands were found when flagella protein, purified by lowering the pH to pH 2 (as recommended by Kobayashi, Rinker and Koffler, 1959) was examined under all these conditions. This is possibly due to the removal of labile amide groups since the bands all migrated faster to the anode than the flagellin itself.

However, homogeneous flagellar protein was not obtained after pH 3 treatment of P. vulgaris NCTC 4175 flagella since the protein was irreversibly denatured when taken to this pH; if the pH of a flagella suspension isolated from this organism was lowered to the point at which the solution just lost its extremely viscous nature, that is to pH 4.5, purified and homogeneous flagellar protein was obtained.

b) Light absorption properties of flagellin solutions

The absorption spectra of P. vulgaris NCTC 100 20 flagellin, both in acid and alkali solution were recorded (Fig. 15). The spectra are typical of a pure protein containing no tryptophan (Beaven and Holiday, 1952).

The values obtained for the extinctions at 215 nm of 1% flagellar protein solutions in water (E_{215} 1%) are given as follows:

Source of flagellar protein	E_{215} 1%
P. vulgaris NCTC 100 20	132
P. vulgaris NCTC 4175	134
P. mirabilis NCTC 6197	125
P. morganii NCTC 232	130
P. rettgerii NCTC 7475	150

This data was occasionally used to calculate the concentrations of flagellar protein solutions (Parish and Ada, 1969).

c) Chemical tests on the purity of flagellins

The values obtained for the content of sugar and phosphate (as inorganic phosphate) in all purified flagellar protein solutions were:

Sugar	less than 0.2%
Phosphate	less than 0.025%

Hence all the tests used to demonstrate the purity of the flagellar proteins isolated showed that they consisted of single protein molecules, that is flagellins. These flagellins were each shown to be homogeneous on electrophoresis in starch and polyacrylamide gels, while chemical tests on the preparations indicated very little sugar or phosphate.

5. Reaggregation of flagellin

a) Reaggregation of flagellin as a means to further purification

When, to further purify preparations, reaggregated material was centrifuged down and reconverted to the flagellin, starch gel electrophoresis showed one band corresponding to the flagellin in question. Reaggregated solutions heated in either 0.05 NH_4HCO_3 at 95° for 30 seconds or 0.1 N NaCl at 60° for 15 minutes readily cleared to give a yellowish

solution, similar in appearance to solutions obtained when proteins did not reaggregate on dialysis.

b) Electron microscopy of reaggregated material

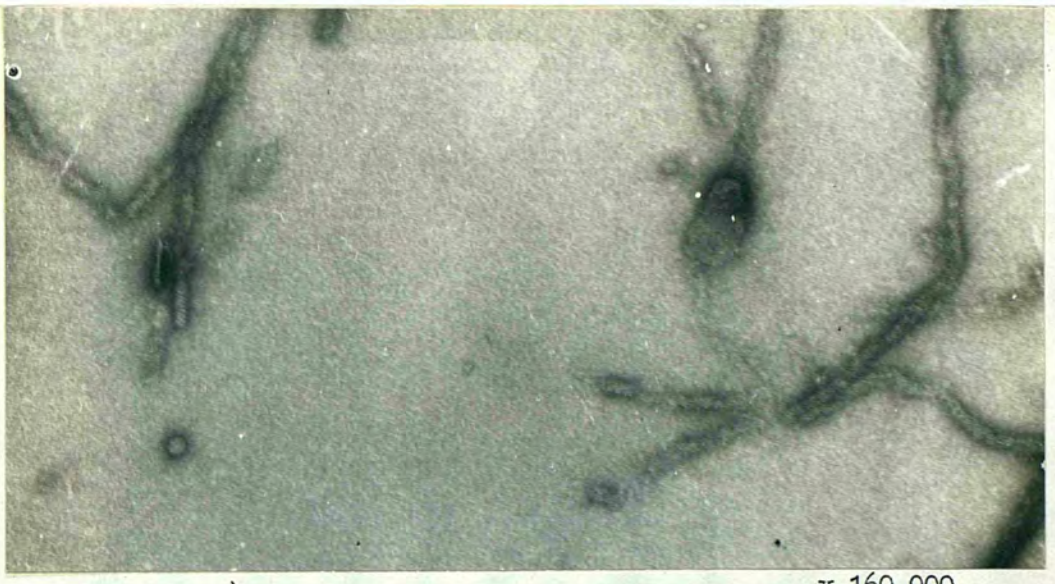
When purified flagellin solutions were dialysed to remove ammonium sulphate, they frequently became viscous and turbid. Such material was examined with the electron microscope. Very often amorphous structures, presumably protein aggregates, were found but sometimes filaments similar in size and surface structure to flagella were present (Fig. 16).

The filaments were short and no wave form could be properly detected. In one instance, following dialysis of a purified preparation of P. vulgaris NCTC 4175 flagellar protein, straight filaments were observed, arising from a central point to give a star-like structure (Fig. 17).

Purified flagellin solutions also reaggregated when the reaggregation method of Wayabayashi, Hotani and Asakura (1969) was used. Flagella-like filaments were routinely observed; again these did not have the wave form of a flagellum assembled in vivo. Seeding of flagellin solutions with flagella fragments was not attempted.

6. Comparative starch gel electrophoresis

For comparison of electrophoretic mobilities in starch gels, flagellins were isolated by removing pure flagella from the top of flagella pellets. Starch gels, run at pH 8.65 in 2M urea, showed only one band for each flagellin. Diagrams representing the comparative starch gels for flagellins isolated from P. vulgaris, P. mirabilis, P. morganii, P. rettgerii, and the Providence group species are given in Figs. 18, 19 and 20. The positions are expressed relative to the migration of P. vulgaris NCTC 100 20 flagellin which equals 1.0. The diagrams show that even within a species the flagellins isolated differed greatly in their electrophoretic mobilities in starch gels.



x 160,000

x 640,000

Fig. 16 P. vulgaris NCTC 100 20 flagellin reaggregated by the method of Wakabayashi, Hotani and Asakura (1969)

Negatively stained with uranyl acetate

Fig. 17 Reaggregated material formed on dialysis of P. vulgaris NCTC 4175 flagellin to remove excess ammonium sulphate x 120,000; negatively stained with uranyl acetate

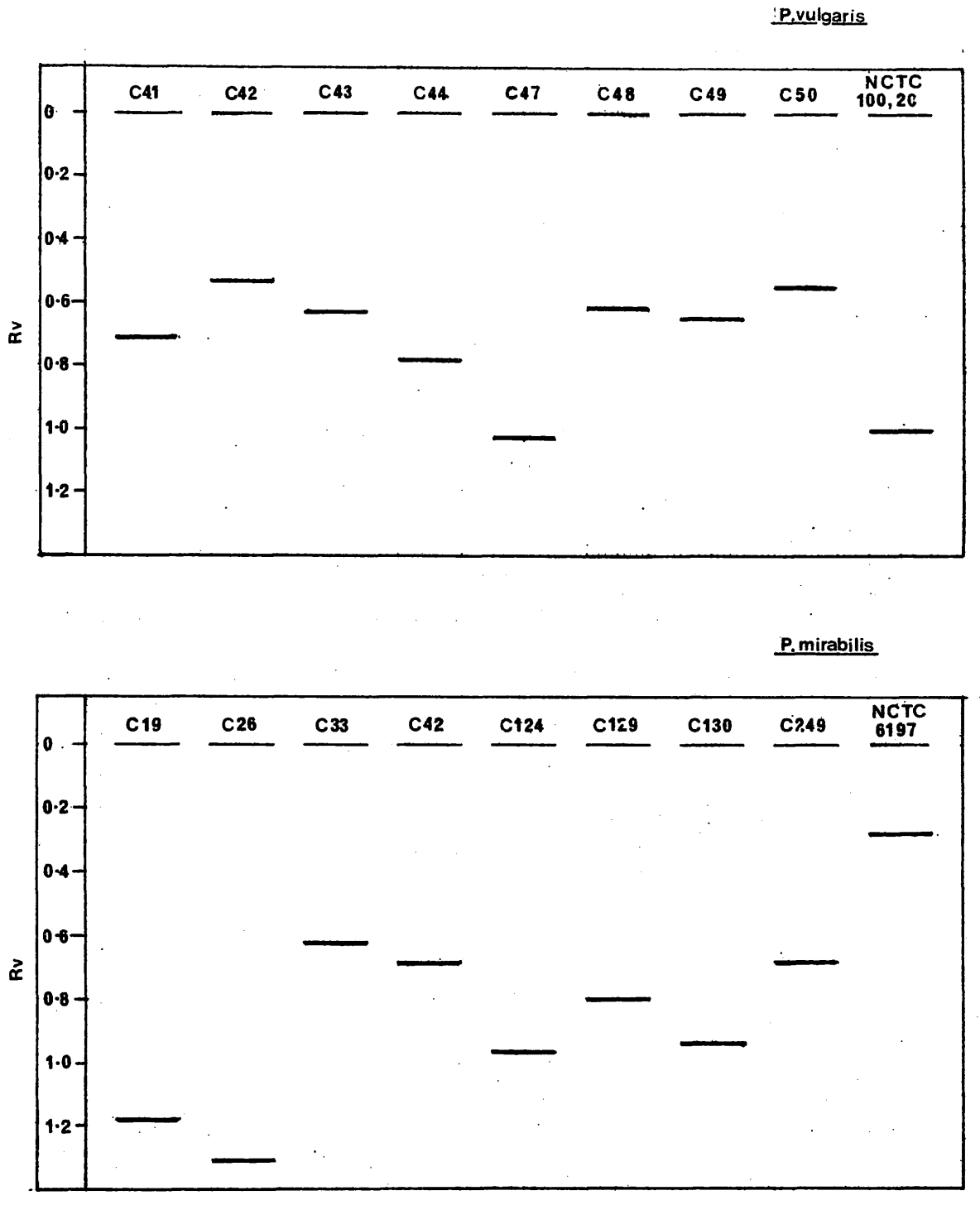


Fig.18 Comparative starch gels of flagellins isolated from the group Proteus - Providencia.

Rv = migration relative to P.vulgaris NCTC 100 20 = 1·0

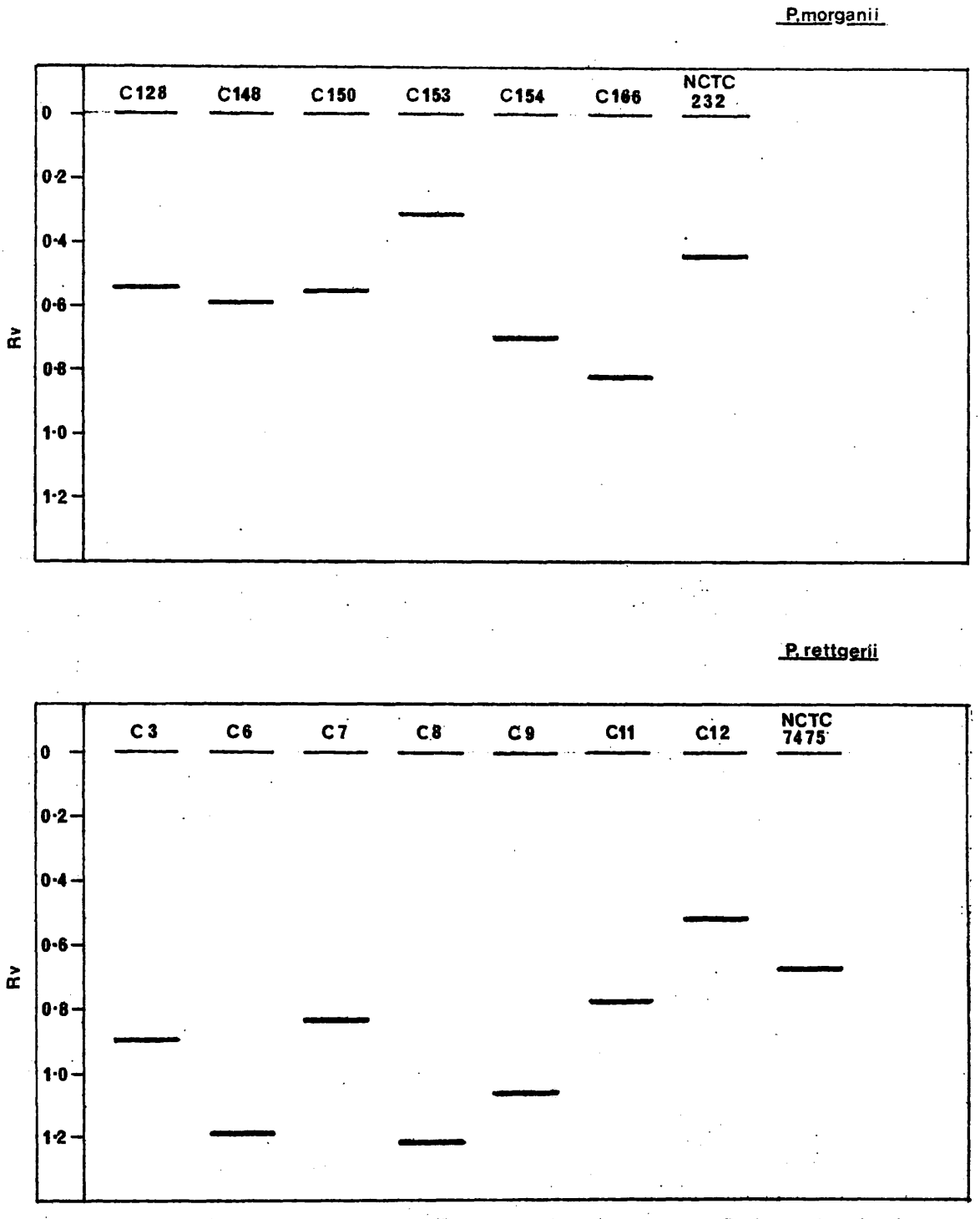


Fig.19 Comparative starch gels of flagellins isolated from the group Proteus – Providencia.

Rv = migration relative to P.vulgaris NCTC 100 20 = 1·0

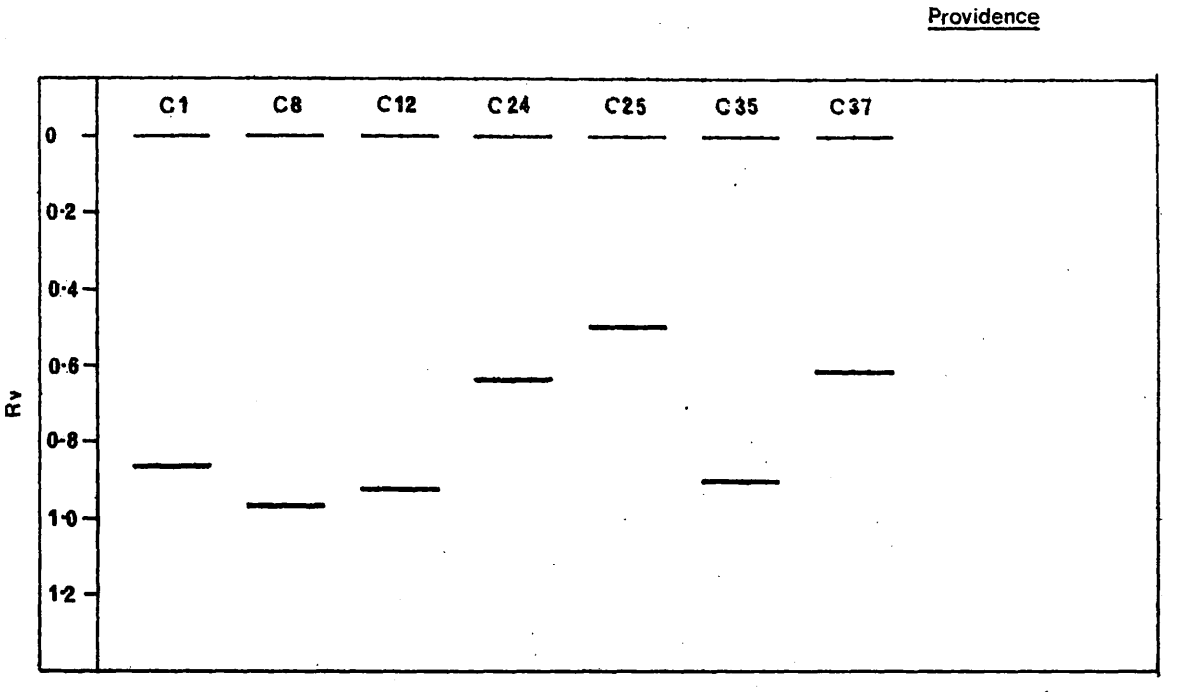


Fig.20 Comparative starch gels of flagellins isolated from the group Proteus – Providencia.

Rv = migration relative to P.vulgaris NCTC 100.20 = 1·0

To test statistically whether there were any correlations in the results, an analysis of variance was performed on the data, comparing the variation within species with the variation between species. A value was obtained for the F ratio of 1.8457; with 35 x 4 degrees of freedom this value is not significant at the 5% level. Hence there is no statistically significant correlation between the flagellins in their electrophoretic mobilities in starch gels.

7. Molecular weight studies

a) Column chromatography

Column chromatography on Sephadex G100, in 0.05M Tris HCl buffer, pH 7.5, 0.1M in KCl, gave good separation of the marker proteins, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome c (Fig. 21). The columns were repoured every week. During this time the elution volumes of the marker proteins decreased slightly, and there was a gradual slowing of the column flow rate.

The elution volumes of each marker protein plotted against the logarithm of their known molecular weight give a sigmoidal curve (Fig. 22). The form of this curve is in agreement with the findings of Fish, Mann and Tanford (1969).

When flagellin, previously dissociated by heating in NH_4HCO_3 or in urea, was applied to the column together with appropriate standards, the elution profile was extremely erratic (Fig. 23). The elution volume of cytochrome c, read at 412 nm, was as expected. However, the absorbance at 215 nm does not correspond to peaks for flagellin, chymotrypsinogen and cytochrome c.

Since columns run in the dissociating solvents 2M urea and 6M guanidine hydrochloride furnished no more information, it was decided to separate the proteins by electrophoresis in polyacrylamide gels

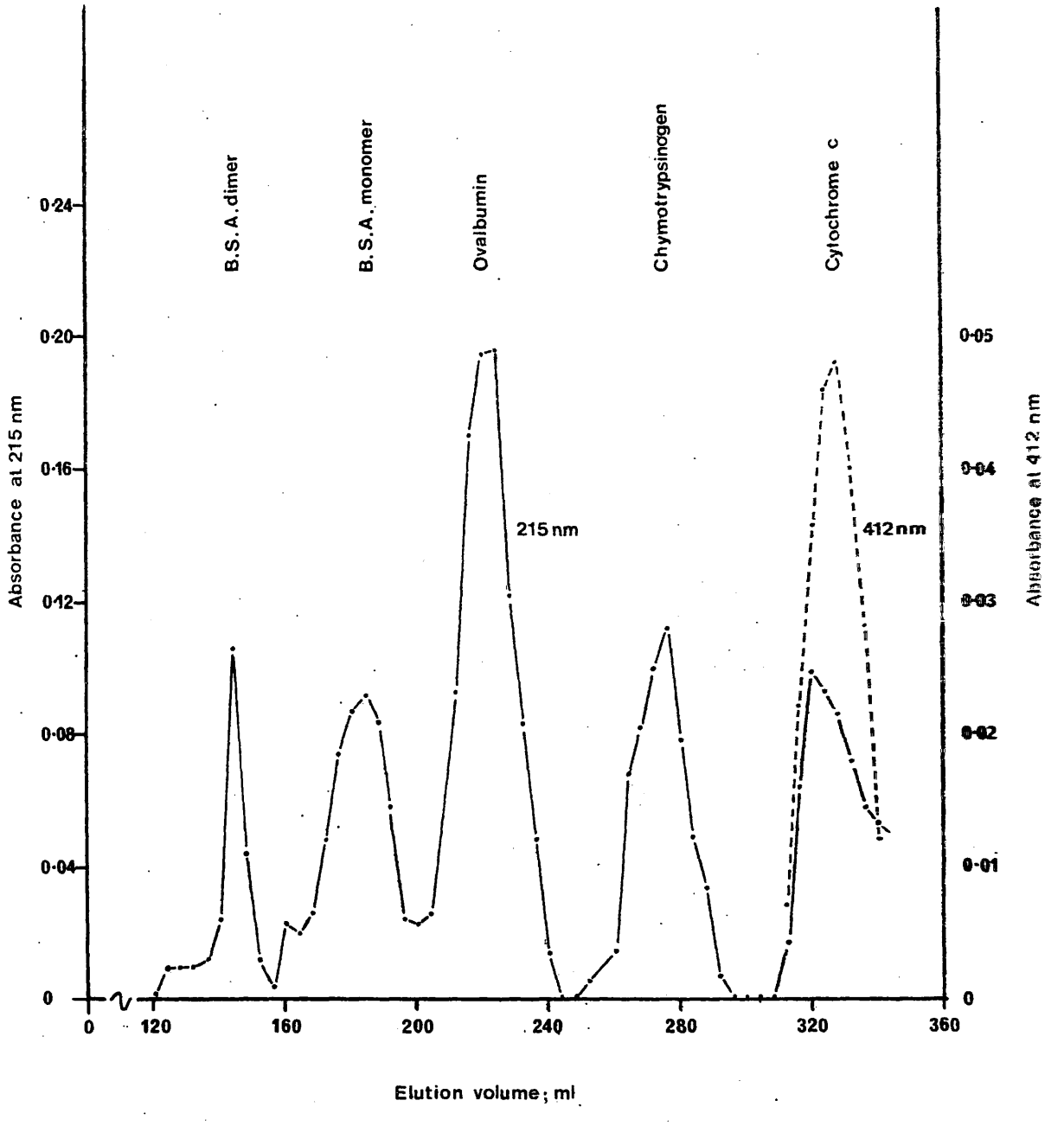


Fig. 21 Elution profile of marker proteins from a 100 cm column of Sephadex G 100.

Sample = bovine serum albumin; ovalbumin; chymotrypsinogen; cytochrome c.

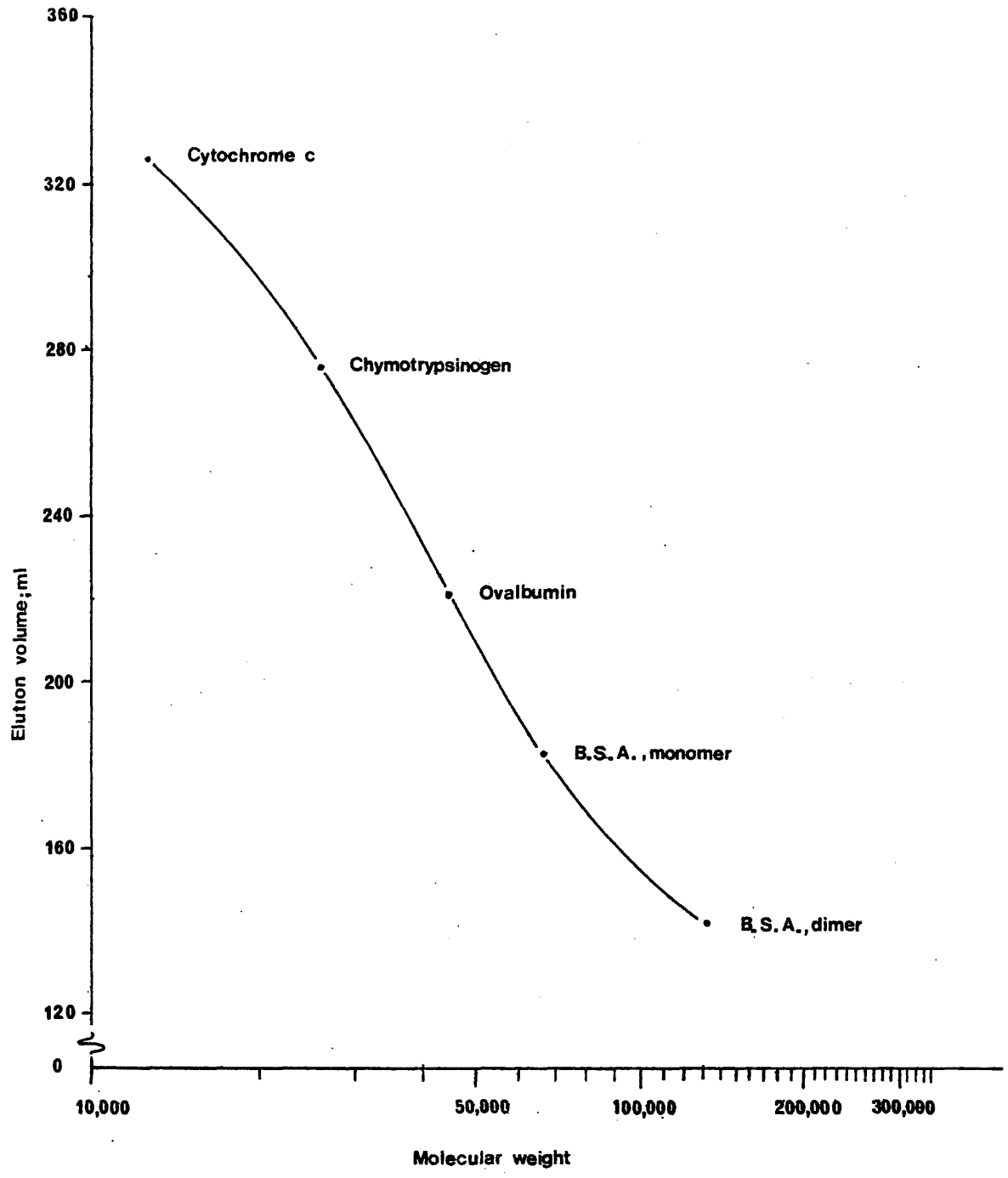


Fig.22 Semilogarithmic plot of molecular weight against elution volume (Ve) for proteins on Sephadex G 100.

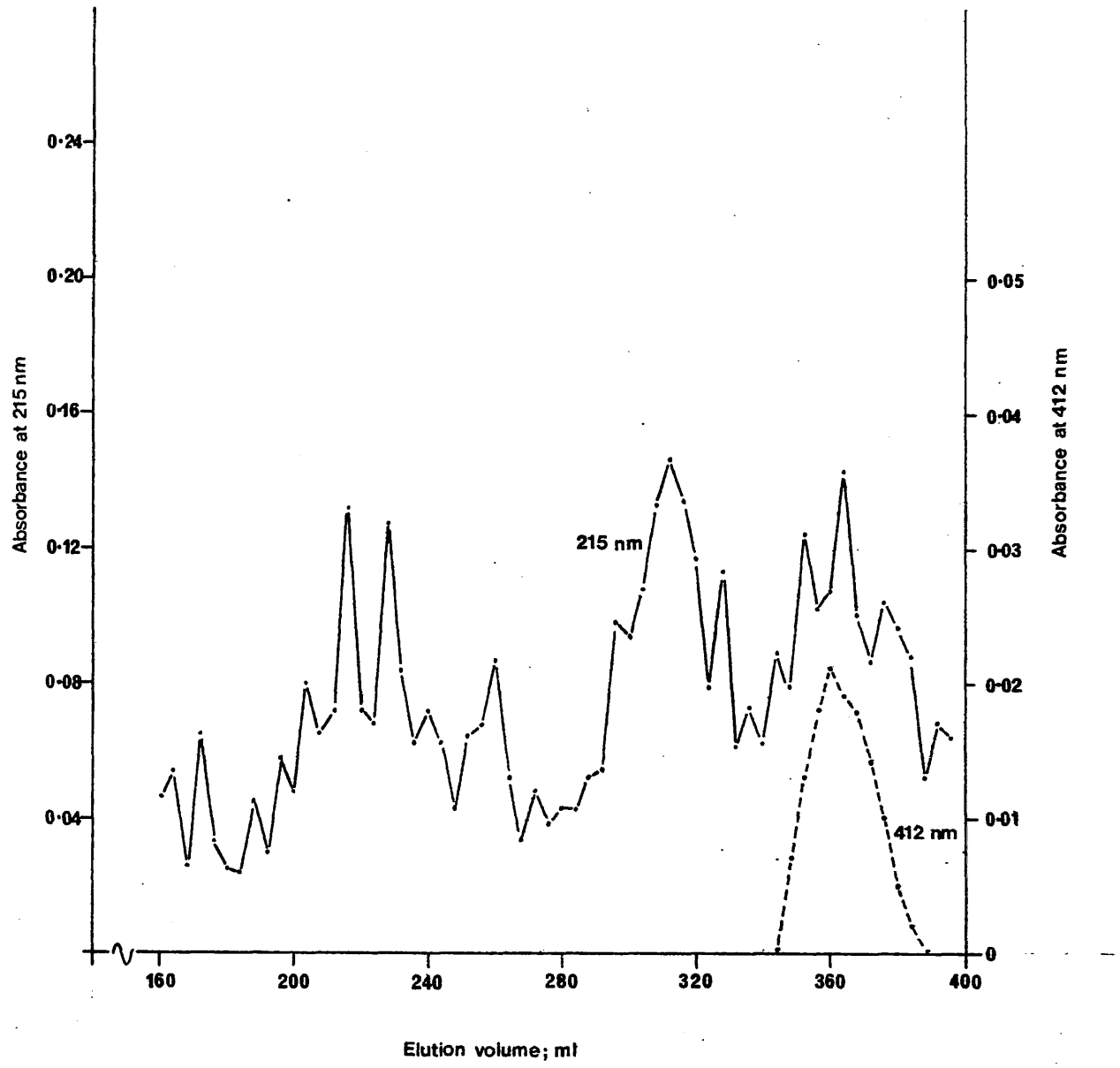


Fig. 23 Elution profile of marker proteins and flagellin from a 100 cm column of Sephadex G100.

Sample = chymotrypsinogen; cytochrome c; P. vulgaris NCTC 100,20 flagellin.

containing SDS. SDS gives all the proteins a net negative charge proportional to the size of the molecule.

b) Polyacrylamide gel electrophoresis

The results given here refer to studies using gels made in pH 8.5 Tris HCl buffer and 0.1% SDS. Preliminary work used SDS phosphate buffer gels (Weber and Osborn, 1969). At this time sulphosalycilic acid was not used to remove excess SDS before phosphate gels were stained, and the SDS precipitated out in the gels. Protein bands in these gels were not visible until several months afterwards. The position of these bands when plotted as distance of protein migration/distance of dye migration (DPM/DDM) against the logarithm of the molecular weight, gave a sigmoidal curve. Similar curves were obtained with marker proteins run in Tris HCl gels containing SDS (Fig. 24). These gels were washed in sulphosalycilic acid to remove the SDS prior to staining. Flagellins ran as single bands in both buffer systems, in the presence and absence of suitable marker proteins. Fig. 25 shows the separation of a mixture of proteins in a Tris HCl gel.

The values obtained for the molecular weights were:

P. vulgaris	NCTC 100 20	37,600 ±	1500
P. mirabilis	NCTC 6197	39,000 ±	1000
P. morganii	NCTC 232	39,600 ±	1000
P. rettgerii	NCTC 7475	44,600 ±	1000
S. typhimurium 1,2		49,500	1 determination

Thus the relative migration positions of all the Proteus flagellins examined correspond to molecular weights of approximately 40,000.

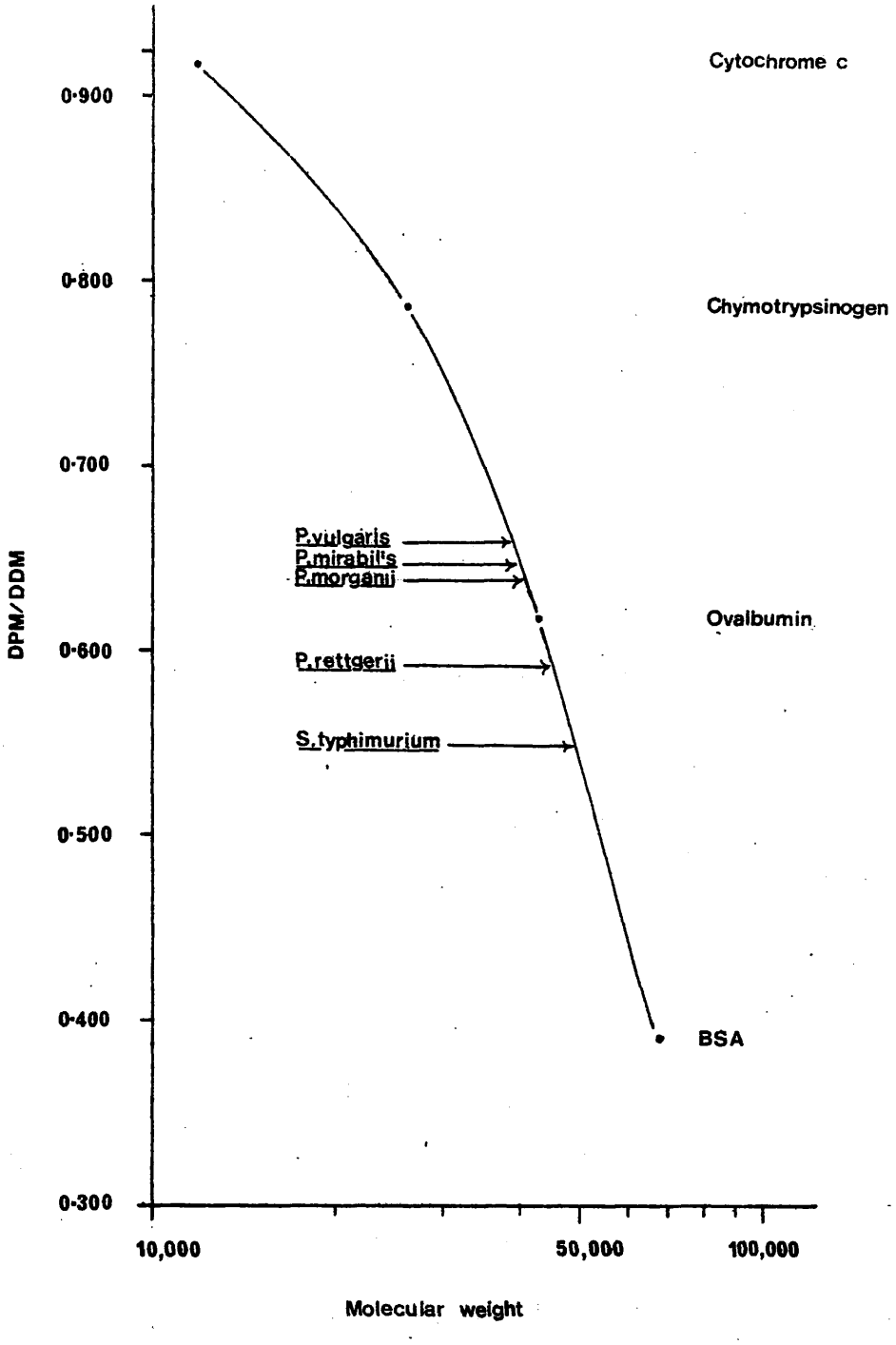


Fig. 24 The relative migration of marker proteins on electrophoresis in polyacrylamide gels (DPM/DDM) plotted against their known molecular weights.

Migration positions of sample flagellins are included on the graph.

Fig. 25 Separation of marker proteins by polyacrylamide gel electrophoresis

Gel buffer: Tris HCl, pH 8.5; 0.1% SDS

8. Amino acid composition

a) Total amino acid analysis

Table I presents typical figures for 24, 48 and 72 hours hydrolyses of P. rettgerii NCTC 7475 flagellin corrected for a molecular weight of 40,000. Corrected figures for serine and methionine were obtained by extrapolation to zero time; threonine appeared to decrease only slightly and a mean figure was taken. Owing to the slow rate of hydrolysis of the adjacent peptide bonds, the amounts of valine and isoleucine increase with time, reaching a maximum between 48 and 72 hours. Hence the final 72 hours figure was used. All other flagellins showed similar differences in serine, methionine, valine and isoleucine values between analyses of 24, 48 and 72 hours hydrolysates, whereas values for other amino acids were in reasonable agreement, irrespective of the time of hydrolysis.

The values obtained from the analysis of five flagellins, isolated from one strain each of P. mirabilis, P. morganii and P. rettgerii and from two strains of P. vulgaris are given in Table II. The figures represent the best values from 24, 48 and 72 hours hydrolyses, calculated on the basis of a molecular weight of 40,000. All flagellins analysed have a high proportion of aspartic and glutamic acids (or amides) threonine and alanine. Little tyrosine, phenylalanine, methionine, proline and histidine is present. Cysteine was never detected. Histidine, not reported in Proteus strains before, was detected in the flagellins of P. vulgaris NCTC 4175 and P. morganii NCTC 232. The amount of proline varied appreciably between the strains studied; also the amounts of methionine (of relevance when cyanogen bromide cleavage is considered). Quantities of tyrosine and phenylalanine present were difficult to establish; it is felt that the figures given represent low estimates of the amounts actually present. This is due to several factors. Tyrosine and phenylalanine are the last amino acids to be eluted off the 150 cm column, and emerge as broad peaks which are difficult to measure accurately.

Amino Acid	24 hours	48 hours	72 hours	Calculated numbers of residues
Lys	27.2	26.0	26.4	27
His	-	-	-	-
Arg	21.4	17.9	18.4	19
Asp	57.5	58.1	57.7	58
Thr	29.1	30.0	28.0	29
Ser	20.4	19.1	16.9	23
Glu	42.2	43.7	44.3	43
Pro	7.1	6.6	6.8	7
Gly	24.6	25.5	25.6	25
Ala	43.6	45.5	45.3	45
Cys	-	-	-	-
Val	24.6	26.8	26.7	27
Met	6.3	5.9	5.0	7
Ileu	17.1	20.5	21.2	20
Leu	33.7	35.9	36.6	35
Tyr	3.5	2.6	3.4	3 (4)
Phe	7.7	6.7	9.1	9 (10)

Table I Amino acid analysis of P. rettgerii NCTC 7475 flagellin

The values given represent the number of residues obtained from analysis of 24, 48 and 72 hours hydrolysates (corrected for a molecular weight of 40,000). For details of correction factors applied see text.

Amino Acid	P. vulgaris NCTC 100 20	P. vulgaris NCTC 4175	P. mirabilis NCTC 6197	P. morganii NCTC 232	P. rettgerii NCTC 7475
Lys	27	34	29	29	27
His	-	2	-	2	-
Arg	12	13	12	16	19
Asp	64	58	65	58	58
Thr	30	27	33	32	31
Ser	27	24	22	21	23
Glu	43	43	38	48	43
Pro	1	5	3	3	7
Gly	29	28	27	26	25
Ala	44	42	37	45	45
Cys	-	-	-	-	-
Val	34	34	28	25	27
Met	2	5	2	3	7
Ileu	22	17	23	18	20
Leu	35	29	33	31	35
Tyr	3	3	3	3	3 (4)
Phe	9	9	9	11	9 (10)

Table II Amino acid analysis of Proteus flagellins

The figures represent the best values from the 24, 48 and 72 hours values, corrected for a molecular weight of 40,000. Other correction factors applied are given in the text.

In addition, there may be some breakdown of tyrosine during acid hydrolysis. In all five strains examined the ratio of phenylalanine to tyrosine is approximately 3:1. There is no appreciable difference in this ratio between those flagellins containing histidine and those not containing histidine.

As a measure of the degree of relatedness between flagellins, the overall differences in amino acid composition of pairs of flagellins have been expressed as Δ (McDonough, 1965). Δ here is taken as the sum of the differences, irrespective of sign, in the number of amino acid residues for each flagellin molecule relative to P. vulgaris NCTC 100 20 flagellin. Ammonia is excluded since the values are not sufficiently reliable; transition between acid and amide is, therefore, not taken into account. Included are values for Δ calculated from published data for P. vulgaris Purdue strain flagellin (Chang, Brown and Glazer, 1969) and S. typhimurium strain SW1061 flagellin (McDonough, 1965).

		Δ
P. vulgaris	NCTC 4175	44
P. mirabilis	NCTC 6197	29
P. morganii	NCTC 232	45
P. rettgerii	NCTC 7475	41
P. vulgaris	Purdue strain	22
S. typhimurium	strain SW1061	65

Table III Summed differences in the number of amino acid residues per flagellin molecule (Δ) relative to the flagellin of P. vulgaris NCTC 100 20

The largest Δ value is that between P. vulgaris NCTC 100 20 and S. typhimurium, strain SW1061. Smaller Δ values are found for P. vulgaris, Purdue strain and P. mirabilis NCTC 6197. However, all of the values are considerably larger than those found by McDonough (1965) for Salmonella antigenically cross reacting flagellins and, in some cases, for non-cross reacting flagellins.

b) The presence of histidine and ∈N-methyl lysine in Proteus flagellins

Since amino acid analysis had revealed histidine in several of the flagellins examined, it was decided to survey the flagellins used for comparative starch gel studies for the presence or absence of histidine. The flagellar proteins, known to be homogeneous, were individually hydrolysed, the hydrolysates spotted on paper and tested for the presence of histidine with the Sakaguchi reagent. Histidine spots were a bright orange pink; tyrosine spots and hence those flagellins not containing histidine, were a brown yellow.

All the four species of Proteus and also the Providence group contained strains which have histidine containing flagellins. However, because the reaction is for both histidine and tyrosine, the results were difficult to interpret. Tyrosine, found in all flagellins so far analysed, complicated the situation. Consequently, some of the hydrolysates were analysed using an amino acid analyser; only the 15 cm column was used. These analyses confirmed the suspected presence of histidine in many of the strains (Table IV).

During the course of these analyses, when hydrolysates of P. morganii C 150, C 153 and C 148 flagellins were examined a peak appeared immediately after lysine and before histidine. This peak compared in position to ∈N-methyl lysine in analyses of Salmonella flagellins. Further tests were carried out to confirm the identity of this amino acid; these

included examination of the hydrolysates, in the presence of suitable markers, by paper electrophoresis at pH 3.5 and by descending chromatography.

It was concluded that ∈N-methyl lysine (NML) was present in these hydrolysates (Table IV). Complete analyses of these proteins have not been carried out but the ratio of NML/lys was about 0.3 compared to about 1.0 for Salmonella flagellins.

Flagellin		∈ N-methyl lysine (mono methyl form)	histidine
Species	Strain		
P. vulgaris	C 60	-	+
"	(NCTC 100 20)	(-)	(-)
"	NCTC 4175	-	+
P. mirabilis	C 249	-	+
"	C 130	-	-
"	(NCTC 6197)	(-)	(-)
P. morganii	C 148	+	-
"	C 150	+	+
"	C 153	+	-
"	C 166	-	+
"	(NCTC 232)	(-)	(+)
P. rettgerii	C 7	-	+
"	C 9	-	+
"	C 12	-	+
"	(NCTC 7475)	(-)	(-)
Providence	C 12	-	+

( ) : NCTC strains; full analyses are given; see Table II

\+ : amino acid present

\- : amino acid absent

Table IV The presence of histidine and ∈ N-methyl lysine in selected flagellins

9. Partial acid hydrolysis

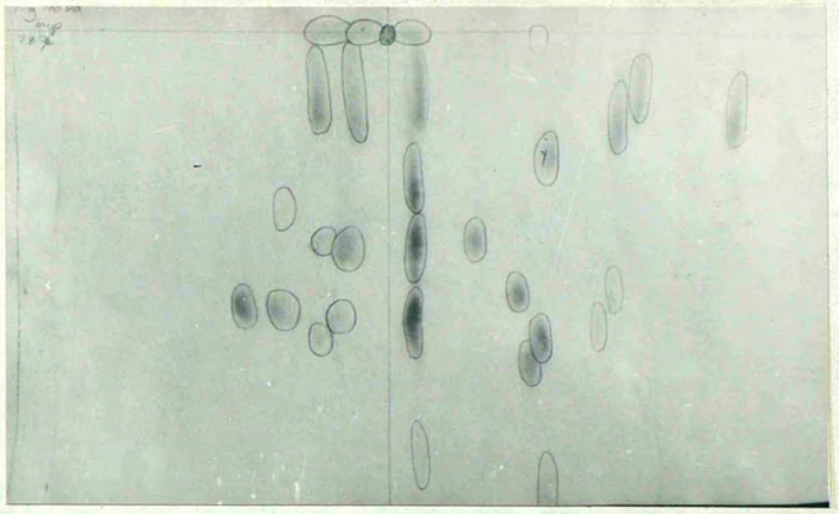
Partial acid hydrolysis of whole flagellar protein of P. vulgaris NCTC 100 20 and the washed "core material" from a tryptic digest revealed differences in pattern when the hydrolysates, after drying down, were subjected to pH 6.3 electrophoresis. This technique was not pursued.

10. Enzymic digestion of flagellins

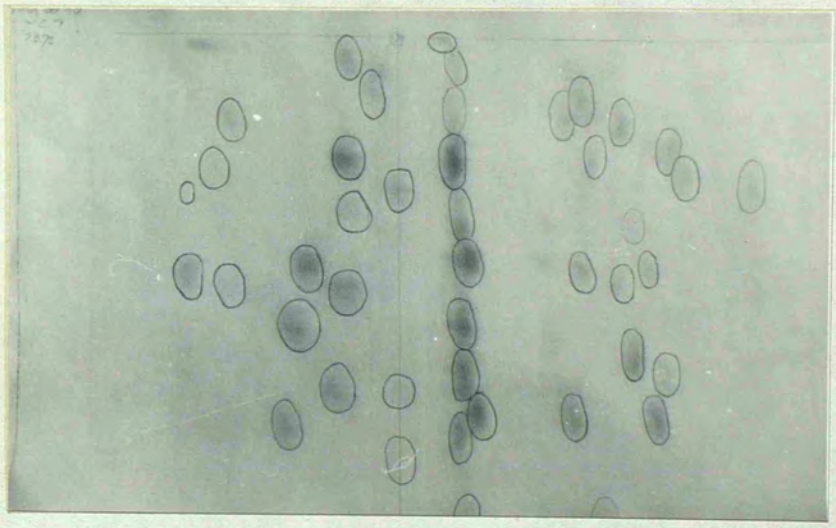
Proteus flagellins were susceptible to enzymic attack. The enzyme most frequently used was trypsin. Flagellin solutions quickly became gelatinous and viscous after the addition of trypsin. In a completed digest a precipitate of "core material" was evident. The precipitate did not spin down easily unless the digest was first frozen and thawed, preferably several times. Digests of flagellin made with chymotrypsin contained less "core material". Digests with pepsin (which is even less specific) contained virtually no precipitate and were not centrifuged prior to spotting for fingerprinting.

a) Tryptic, chymotryptic and peptic maps

When digests made under controlled conditions were spotted on chromatography paper and subjected to descending chromatography followed by electrophoresis, very reproducible peptide maps were obtained (Fig. 26). Comparative tryptic fingerprint maps were made for P. vulgaris NCTC 100 20; P. vulgaris NCTC 4175; P. mirabilis NCTC 6197; P. morganii NCTC 232 and P. rettgerii NCTC 7475 flagellins (Figs. 27, 28, 29). If tryptic peptide maps of flagellins were compared visually, generally about a dozen spots were more or less similar in their chromatographic and electrophoretic mobilities. Slight changes in the position of such spots between different flagellins can be attributed to the appreciable effect that substitution of one amino acid for another can have on the mobility of a peptide. The extent of the effect of this substitution depends on the amino acid substituted.



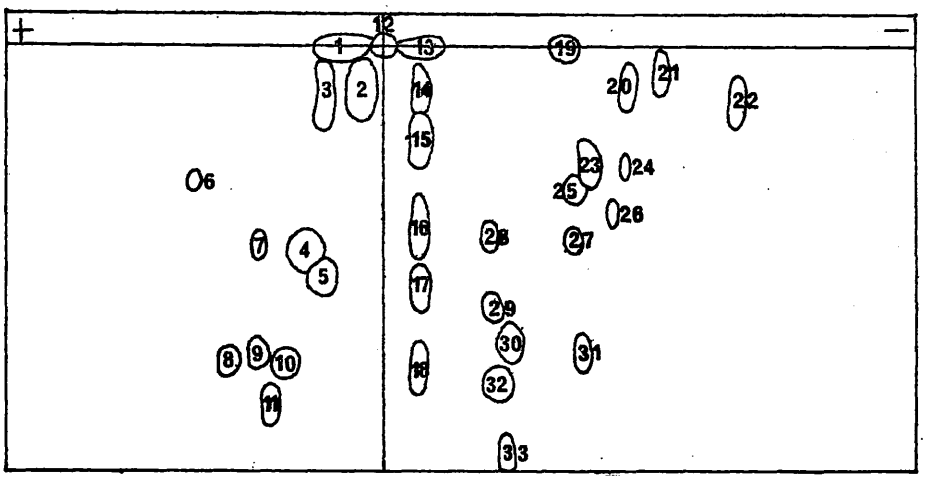
a) tryptic digest



b) chymotryptic digest

Fig. 26 Enzymic digestion of P. vulgaris NCTC 100 20 flagellin

a) peptide reference numbers



b) arginine and tyrosine peptides

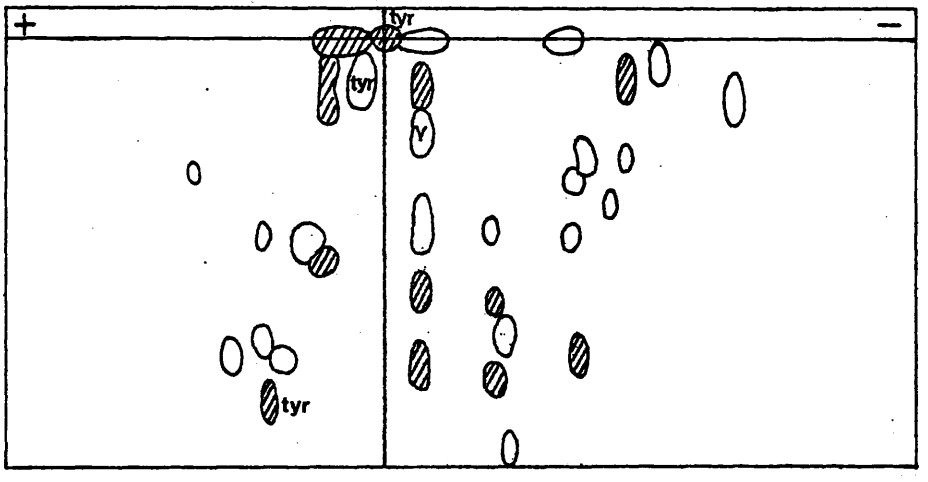


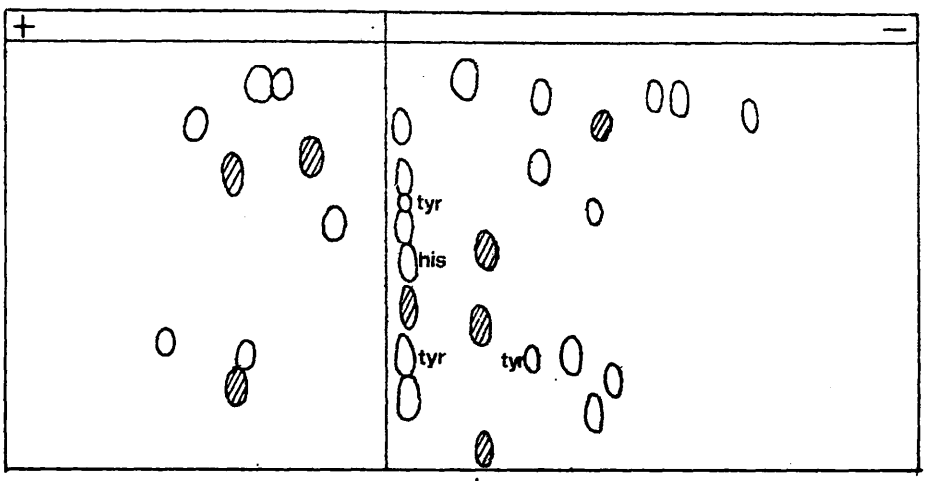
Fig. 27 Tryptic peptide maps of P. vulgaris NCTC 100 20 flagellin.

⊘ - peptides containing arginine

tyr- peptides containing tyrosine

Y - peptides with N terminal glycine or amide residue

P. vulgaris NCTC 4175



P. mirabilis NCTC 6197

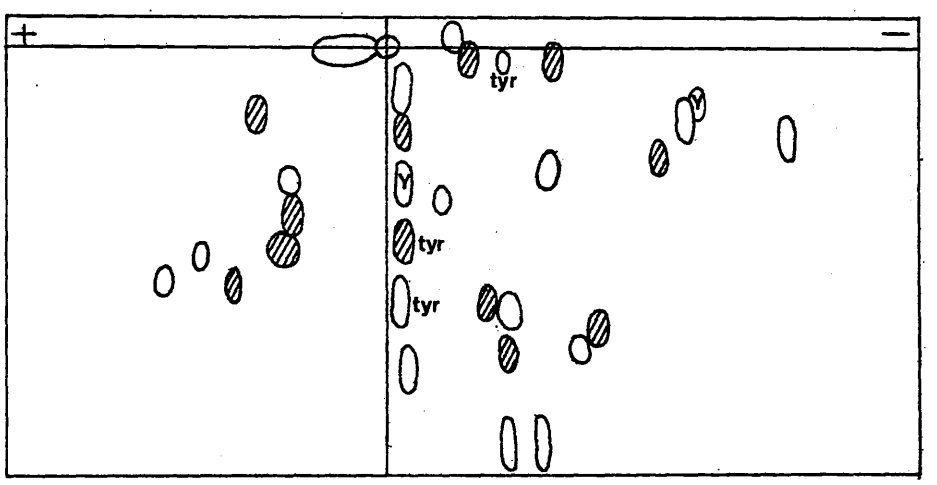


Fig. 28 Tryptic peptide maps.

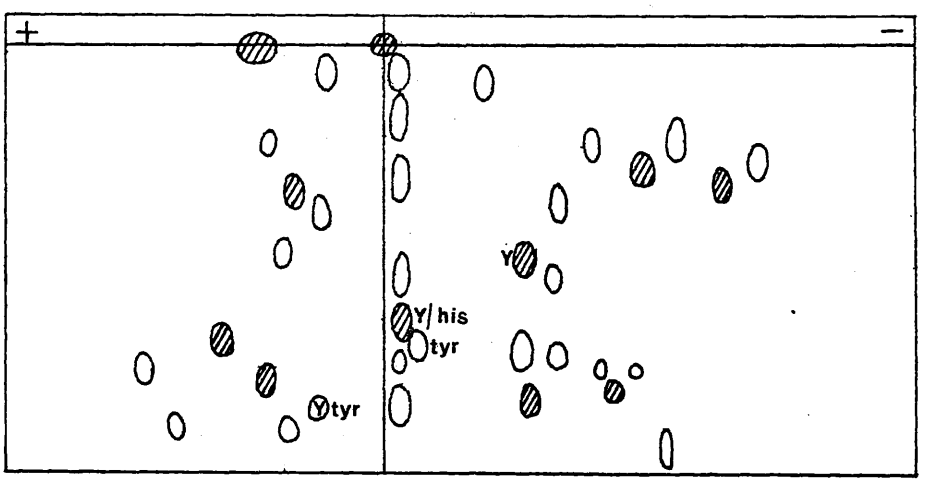
◍ – peptides containing arginine

tyr – peptides containing tyrosine

his – peptide containing histidine

Y – peptide with N terminal glycine or amide residue

P.morganii NCTC 232



P.rettgerii NCTC 7475

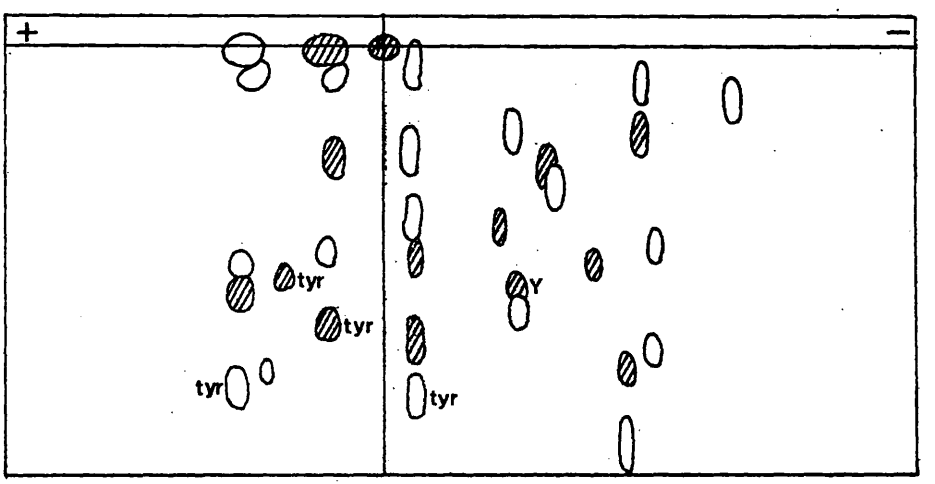
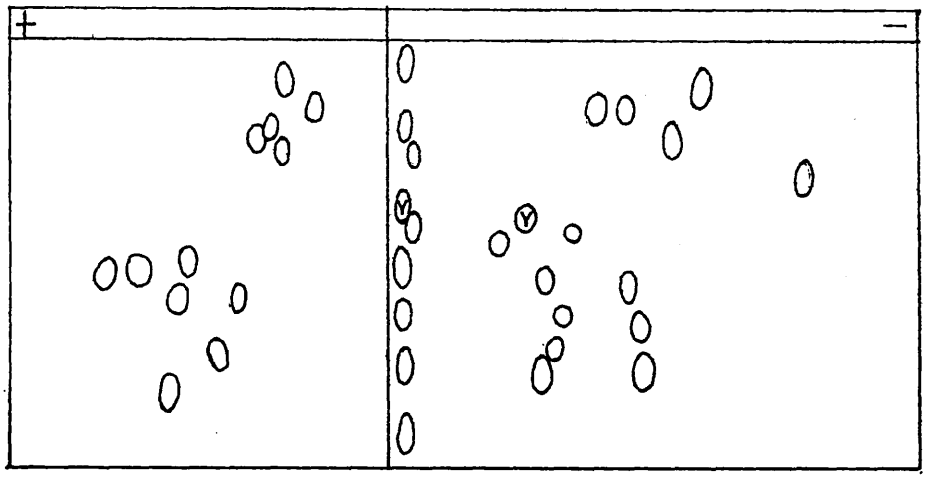


Fig. 29 Tryptic peptide maps.

◍ -peptides containing arginine
tyr-peptides containing tyrosine
his-peptide containing histidine
Y -peptides with N terminal glycine or amide residue

Chymotryptic map



Peptic map

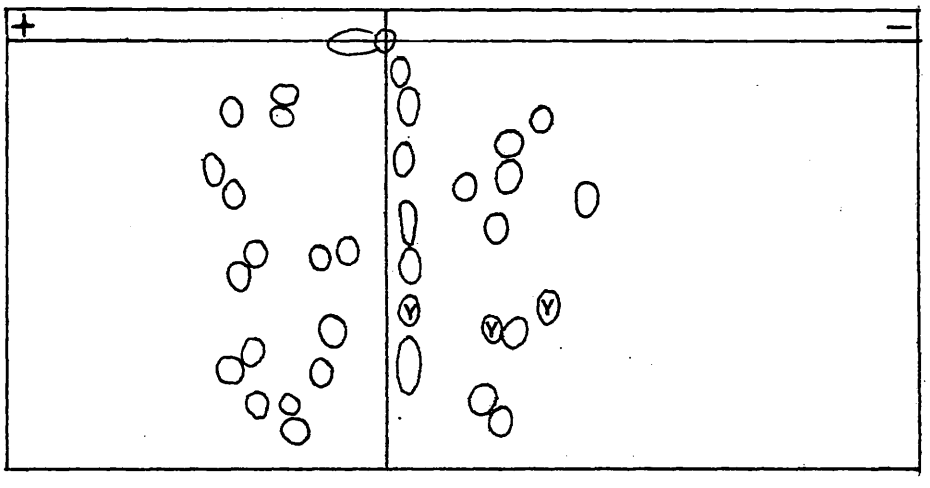


Fig.30 Chymotryptic and peptic maps of P. vulgaris NCTC 100 20, flagellin.

Y - peptides with N terminal glycine or amide residue

The peptide maps of P. vulgaris NCTC 100 20 and P. mirabilis NCTC 6197 flagellins were also compared by mixing digests of the two, spotting, and running as before. In this instance at least 64 different spots were distinguished; some of the spots were separated, however, by very small distances.

b) Peptides containing specific amino acids

Tests, specific for certain amino acids, were used to further characterise the peptides. The numbers of tyrosine and histidine containing peptides were in reasonable agreement with the numbers of residues obtained by amino acid analysis (Table V). Perfect agreement is to be expected if the similar residues are not adjacent on the polypeptide chain but distributed through the molecule and are not contained within the "core material". Generally the numbers of arginine containing peptides were slightly fewer than the numbers of arginine residues indicated by amino acid analysis. Trypsin cleaves protein chains on the carboxyl side of either lysine or arginine residues. Hence the number of arginine residues should equal the number of arginine containing peptides (unless arginine is the C terminal amino acid). It is of note that when tryptic digests are made, such insoluble "core material" results. This "core material" possibly contains portions of the "core material" sequence containing, for example, the missing arginine residues.

Also shown in Table V are the total numbers of regularly recorded peptides for tryptic peptide maps and the summed numbers of lysine and arginine residues as determined by amino acid analysis. In all cases the number of peptides found are slightly fewer than the numbers theoretically expected. If trypsin cleaves flagellin molecules on the carboxyl side of every lysine or arginine residue the number of peptides found should equal the sum of the lysine and arginine residues + 1. In all cases the numbers of peptides found are slightly fewer than the numbers theoretically expected; some may be accounted for by the "core material". Chymotryptic and peptic maps were only made for the flagellin of P. vulgaris NCTC 100 20.

Source of flagellin	P. vulgaris NCTC 100 20	P. vulgaris NCTC 4175	P. mirabilis NCTC 6197	P. morganii NCTC 232	P. rettgeri NCTC 7475
Number of arginine residues	12	13	12	16	19
Number of arginine peptides	12	8	12	11	14
Number of tyrosine residues	3	3	3	3 (4)	3 (4)
Number of tyrosine peptides	3	3	3	2	4
Number of histidine residues	-	2	-	2	-
Number of histidine peptides	-	1	-	1	-
Total number of peptides	33	32	32	38	33
Total of lys + arg residues	37	47	39	45	43

Table V Numbers of tryptic peptides derived from the tryptic digestion of Proteus flagellins containing certain amino acids

The lesser specificities of chymotrypsin and pepsin should result in far more peptides than for tryptic digestion and this was confirmed (Fig. 30).

11. Carboxy-terminal sequences

When Salmonella flagellins are treated with carboxypeptidase A-DFP (which will not release basic amino acids) no amino acids are found when the supernatants are chromatographed to identify any amino acids present. However, treatment with carboxypeptidase B-DFP results in the release of arginine, leucine and a small amount of serine. Arginine and lysine, when analysed quantitatively, are released in the proportions of about 1:2; arginine is the first amino acid released. Hence this amino acid is C terminal (McDonough; unpublished results).

Proteus flagellins were similarly treated to see if they resembled the Salmonella flagellins in their C terminal region.

a) Amino acids released by carboxypeptidases A and B

No amino acids were released when P. vulgaris NCTC 100 20 flagellin was treated with carboxypeptidase A-DFP. Carboxypeptidase B-DFP digestion of P. vulgaris NCTC 100 20, P. vulgaris NCTC 4175, P. mirabilis NCTC 6197, P. morganii NCTC 232 and P. rettgerii NCTC 7475 flagellins for 1.5 hours, released arginine and leucine. These amino acids were detected by paper electrophoresis at pH 1.5 and chromatography of supernatants after the remaining protein had been precipitated out. Serine was also detected, as a slightly fainter spot.

With respect to these experiments it is worth noting that, in contrast to Salmonella flagellins, the pH at which Proteus flagellar protein in water would precipitate with acetone was very critical. A very small change in pH (less than 0.2 units) allowed a solution of flagellar protein to precipitate when acetone was added.

b) Quantitative analysis of amino acids released by carboxypeptidase B-DFP

Supernatants resulting from the carboxypeptidase B-DFP digestion were also subjected to quantitative amino acid analysis. Peaks corresponding to arginine, leucine, and for longer periods of digestion serine, were found. Other trace amounts of amino acids were present; these increased slightly with longer periods of digestion. It is likely that these trace amounts of amino acids produced are a result of residual endopeptidase activity still present in the carboxypeptidase B-DFP preparation.

Typical results were:

P. vulgaris NCTC 100 20 flagellin — Digested with carboxypeptidase (4 hrs.)

Amino acid analysis of the supernatant

	µ Moles
Arginine	0.065
Leucine	0.148
Serine	0.063

These figures give a ratio of 1.00 : 2.28 : 0.97. Knowing that arginine is the C terminal amino acid and that serine is released after the leucine which does not further increase relative to arginine, the C terminal sequence is probably ... ser - leu - leu - arg COOH

c) Identification of the C terminal peptide

Fingerprint maps were made for tryptic digests of carboxypeptidase B treated flagellins of P. vulgaris NCTC 100 20; P. vulgaris NCTC 4175; P. mirabilis NCTC 6197; P. morganii NCTC 232; and P. rettgerii NCTC 7475. No change in the position or removal of any peptide relative to the untreated control was noted in the maps of the tryptic digests of

carboxypeptidase treated proteins.

12. Amino-terminal sequences

a) The N terminal amino acid of Proteus flagellins

Dansylation of flagellar protein of P. vulgaris NCTC 100 20; P. vulgaris NCTC 4175; P. mirabilis NCTC 6197; P. morganii NCTC 232 and P. rettgerii NCTC 7475, followed by the hydrolysis and separation of the hydrolysis products by pH 1.9 electrophoresis, demonstrated that alanine was the N terminal acid for all the flagellins examined.

b) Tentative N terminal amino acid sequences of flagellins

N terminal sequence studies using the method of Weiner, Platt and Weber (1972) were only partially successful. P. vulgaris NCTC 100 20 dansylated flagellin precipitated easily when trichloracetic acid was added. Excess dansylic acid was left in solution. However, flagellin isolated from P. rettgerii NCTC 7475 would not precipitate with this treatment and, for this flagellin, the step was omitted.

No difficulties were found in the separation of a standard mixture of amino acids according to the methods of Woods and Wang (1967) and Weiner, Platt and Weber (1972). When hydrolysates of the sequentially degraded, dansylated flagellar protein were examined the results were not very clear. Many breakdown products formed in spite of the great care taken. In addition the method was not as sensitive as hoped; even when comparatively large amounts of flagellar protein (about 1 mg) were used, these were insufficient to determine more than a few residues.

The tentative N terminal sequences for the flagellins examined are:

	N terminal	C terminal
P. vulgaris NCTC 100 20	ala, leu, val, gly ...	
P. rettgerii NCTC 7475	ala, leu, val, gly ...	

13. Fractionation and sequence of peptides

a) Fractionation and analysis of tryptic and chymotryptic peptides

A typical elution profile of a tryptic digest of P. vulgaris NCTC 100 20 flagellin from a column of Dowex 50x2, assayed with ninhydrin, is shown in figure 31. Individual peaks when pooled and evaporated down gave the electrophoretic pattern shown. As expected, the acidic peptides were eluted early and the basic peptides late. Neutral peptides were eluted all the way through the fractionation. The sequence of peptide elution was easily reproduced. Chymotryptic peptides gave a similar pattern when eluted from a similar column. However, chymotryptic peptides were not studied further.

The peptides of the pooled peaks were separated by preparative electrophoresis. Chromatography often was used to further purify the peptides since most of the electrophoretic bands consisted of more than one peptide.

b) Characterisation and sequence of tryptic peptides

The peptides purified, their positions on a peptide map, their amino acid composition as identified by paper chromatography and, if known, their sequence identified by Edman degradation followed by dansylation, are given in Table VI.

Peptide amino acid compositions were easily found by subjecting hydrolysates to a combination of electrophoresis at pH 1.9 and ascending chromatography (Fig. 32). It was not possible to carry out quantitative amino acid analyses at the time the peptides were examined. The qualitative amino acid composition of the peptides correlate with the positions of the peptides on peptide maps. For

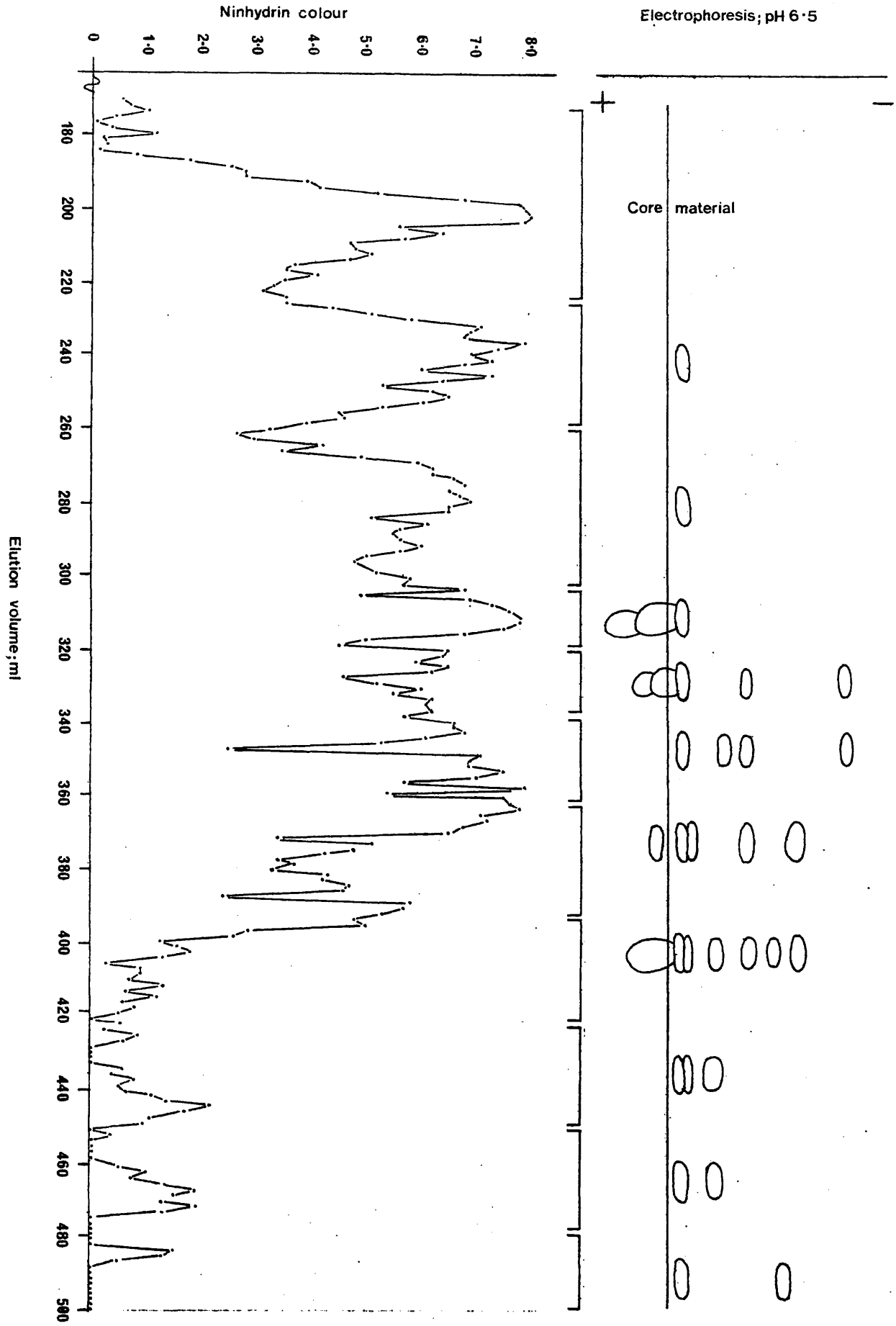
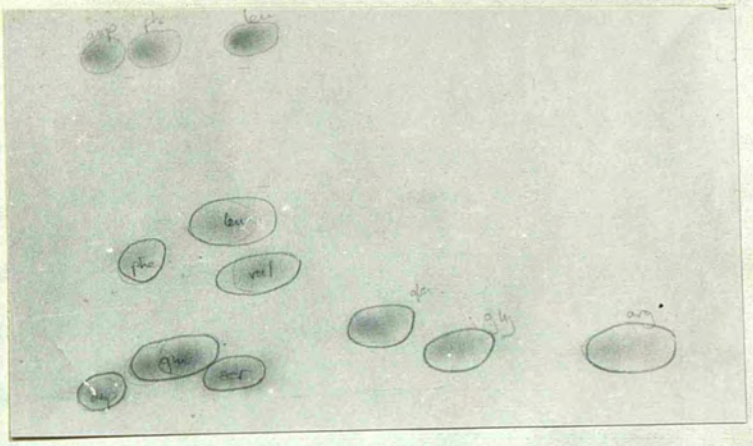


Fig. 31 Separation of a tryptic digest of P. vulgaris NCTC 100 20 flagellin; elution profile from a column of Dowex 50 and electrophoresis of the peaks obtained.

Peptide	Corresponding peptide on tryptic map	Amino acid composition (leu = leu/ileu)	Sequence (if known) NH_2.....COOH
A	8, 9 or 10	lys,gly,ala,val,phe,asp.	phe-asp-val-ala-(gly...)-lys
B	15	lys,gly,ala,val,glu,asp,tyr.	asp-tyr-val-(gly,ala,glu)-lys
C	16	lys,gly,ala,val,glu.	(gly,ala,val,glu)-lys
D	18	arg,leu,ala,glu.	leu-ala-glu-arg
E	21	lys,ser	ser-lys
F	22	lys	lys
G	29	arg,gly,ala,val,leu,ser,glu,asp,phe	phe-ala-glu/asp-leu-(gly,val,ser,glu/asp)-arg
H	30	lys,gly,ala,val,glu,ser.	val-gly-ser-glu-(..ala..)-lys
I	32	arg,gly,ala,val,leu,ser,asp.	ileu-gly-ser-ala-(val,asp)-arg

Table VI The position, amino acid composition and sequence (if known) for tryptic peptides of P. vulgaris 100 20 flagellin.

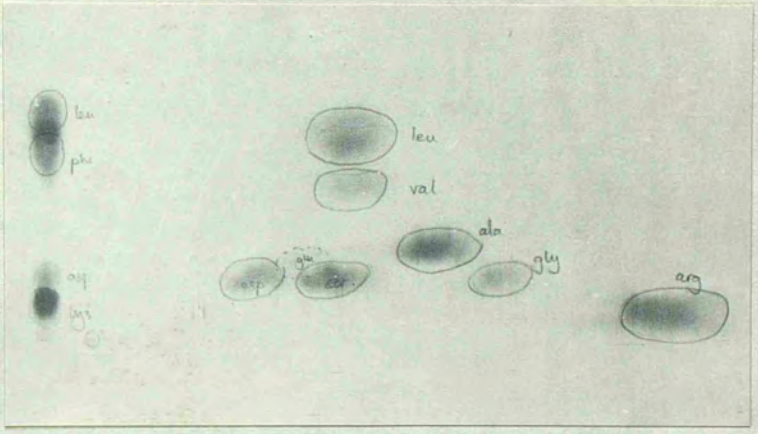


Hydrolysate of peptide 29 (G)

(1)

+ ←——— Electrophoresis ———→ -

(2) Chromatography



Hydrolysate of peptide 32 (I)

Fig. 32 Amino acid fingerprint maps

Hydrolysates of tryptic peptides isolated from a digest of P. vulgaris NCTC 100 20 flagellin

example, those peptides with proportionally more leu/ileu ran faster chromatographically than peptides lacking these amino acids.

The sequences given represent the amino acids identified as N terminal after repeated sequential Edman degradation of the peptide followed by dansylation. In all cases the initial N terminal amino acid of the peptide was unequivocally identified. However, after 2-3 cycles of Edman degradation many breakdown products began to appear, although the utmost care was taken to try and prevent this. Hence further sequencing of the peptide was not possible.

To a certain extent the complete sequences of peptides can be obtained by extrapolation. Since lysine or arginine is the C terminal amino acid, in a peptide derived from tryptic digestion this amino acid can be fitted into its correct position at the end of the sequence. Then, if only one amino acid, known to be present, is unaccounted for, this can be inserted in the sequence between those amino acids of known sequence and the C terminal lys or arg residue. If, however, there are two amino acid residues in the peptide not placed in sequence, this extrapolation is not possible. In addition, one has to be certain that there is only one residue of any one amino acid unaccounted for in the sequence before the sequence can be extrapolated.

14. Maleylation or citraconylation of flagellin

Proteus flagellins easily became insoluble when broken down either chemically, for example using cyanogen bromide, or enzymically. To circumvent this problem, flagellins were often maleylated or citraconylated prior to breakdown or after breakdown to render the products soluble.

P. vulgaris NCTC 100 20 flagellin was readily maleylated with maleic anhydride or citraconylated with citraconic anhydride. When citraconylated protein was digested with trypsin no "core material" was visible in the solution. In contrast, when unblocked, previously citraconylated, flagella protein was digested copious "core material" was found. Fingerprint maps of tryptic digests of the citraconylated flagellin either digested as the citraconylated protein or unblocked by low pH treatment before digestion showed great differences. Only 14 peptides, and a slight trace of insoluble "core material" were found for trypsin digested citraconylated protein. This contrasts with the usual 33 peptides plus copious "core material", obtained by tryptic digestion of untreated flagellin or unblocked, previously citraconylated flagellin.

A tryptic digest of the citraconylated flagellin was separated on a 100 cm column of Sephadex G75 in 0.1N NH_4HCO_3. The peaks obtained were evaporated down, unblocked and gave a series of peptides on electrophoresis at pH 6.5. The next step is the separation and characterisation of these fragments. This could include amino acid analysis of the fragments and their splitting by various enzymes to give several peptides.

15. Cyanogen bromide cleavage

In an attempt to obtain large fragments of the various flagellins examined, flagellar protein was reacted with cyanogen bromide. Completed digests appeared very viscous and opaque. If the digests were briefly dialysed to remove excess reagents they remained in this state and were difficult to solubilise. However, cyanogen bromide digests which were directly freeze-dried were readily soluble in 0.2N acetic acid; only a slight trace of insoluble material remained. The freeze dried cyanogen bromide digests were also soluble after citraconylation; again very little insoluble material remained.

a) Polyacrylamide gel electrophoresis of digests

Electrophoresis in pH 2.3, 6M urea polyacrylamide gels, or in pH 8.5, 0.1% SDS polyacrylamide gels, revealed a series of faster running bands relative to sample flagellins. Cyanogen bromide digests of P. vulgaris NCTC 100 20 flagellin run in pH 2.3 gels gave a few faint bands in addition to a band corresponding to the parent flagellin. However, digested flagellin of P. rettgerii NCTC 7475, run in this system, gave a whole series of strong bands running faster than the flagellin. It is of note that the P. rettgerii flagellin used in the present study had 7 methionine residues while P. vulgaris NCTC 100 20 flagellin has only two.

b) Separation of digests on Sephadex

Freeze dried digests, taken up in 0.2N acetic acid or citraconylated to solubilise, gave a series of peaks (assayed by the method of Moore and Stein, 1954) when chromatographed on columns of Sephadex G75 run in 0.2N acetic acid. These peaks were shown to consist of various components when samples, concentrated by evaporation, were applied to pH 2.3, 6M urea gels. Each peak contained one or more

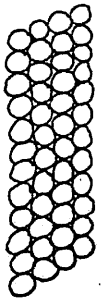
bands corresponding to those found on polyacrylamide gel electrophoresis of the complete digest.

As yet no attempt has been made to further purify the fragments obtained as a result of cyanogen bromide cleavage and subject these fragments to preliminary sequence studies.

DISCUSSION

The flagella of the Proteus species and strains studied were long and sinuous, with three to four wavelengths per flagellum. In agreement with Hoeniger (1965) curly flagella were observed when cells were grown in liquid media at pH 5.0. The P. vulgaris, P. mirabilis, P. morganii and P. rettgerii strains examined did not possess flagella sheaths, although amorphous mucilaginous material was often present round their flagella. This contrasts with the findings of Lowy and Hanson (1965) who found mucilaginous sheaths on the flagella of an unspecified strain of P. vulgaris.

The surface of negatively stained flagella examined with the electron microscope appeared either "beaded" or "lined" (figure 33); both types of surface appearance were often seen on the same flagellum. Generally, three to four "beads" or "lines" were seen across the width of the flagellum. Abram, Vatter and Koffler (1966) have also reported that, when Bacillus stearothermophilus cells are examined, both types of the surface appearance can be seen along the one flagellum. However, Lowy (1965) has reported either a "beaded" or a "lined" surface appearance on Proteus flagella; frequently the flagella surface was neither "beaded" or "lined". Lowy and Spencer (1968) have suggested that the surface appearances of "beaded" or "lined" flagella seen in the electron microscope reflect differences in the structure of the sub-units but that the geometrical arrangement of these sub-units is essentially the same in all types of flagella.



"Beaded" appearance

"Lined" appearance

Fig. 33 Diagrams of the appearances of negatively stained P. vulgaris flagella at high magnification

The examination of negatively stained flagella with surface "beaded" appearance in the electron microscope has indicated an axial periodicity of 52 Å for both P. vulgaris flagella and the flagella of many other genera. The presence of a 52 Å periodicity can also be deduced from the X-ray diffraction pattern given by the flagella of P. vulgaris (Champness, 1971). Comparison of the X-ray diffraction patterns for the "lined" flagella of B. pumilus and the "beaded" flagella of S. typhimurium (Champness and Lowy, 1968) reveals slight differences which suggest that the structure of the sub-units is different for the two forms. No published data concerns the differences between "beaded" and "lined" portions of the flagella of P. vulgaris.

Structures which appeared as cross sections consisting of the negatively stained broken ends of flagella of P. vulgaris NCTC 100 20 presented six sub-units arranged in two groups of three (see Figure 34).

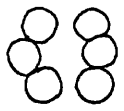


Fig. 34 Diagram of the cross section of the flagellum of P. vulgaris

Various numbers of sub-units (5-8) have been indicated in cross sections of Salmonella flagella. Kerridge, Horne and Glauert, (1962) have examined cross sectional material which indicated five sub-units; Lowy and Hanson (1965) have proposed a model in which eight sub-units would be evident in cross section. The Kerridge, Horne and Glauert (1962) models for the flagellum consist of either three helically wound components or five strands of sub-units arranged parallel to the axis of the model. Lowy and Hanson (1965) have proposed a model that consists of eight longitudinal rows of spherical units close packed to form a

cylindrical shell. Recently, O'Brien and Bennett (1972) have made a study of the straight flagella of a mutant of S. typhimurium using electron microscopy and optical diffraction and filtering. They consider that the flagellum is constructed from a single helix; in their model this helix shows eleven sub-units in cross section. These sub-units are probably extended into the centre of the flagellum. Bode, Engel and Winklmair (1972) have shown that the flagellar sub-units are ellipsoidal in form.

Interpretation of the possible cross sections obtained in the present study can tentatively be elucidated with the aid of a model constructed of two helically wound strands (Figure 35). A broken flagellar fragment end, as seen in the present study, would correspond to an approximate cross section. Such a cross section could show six sub-units in the plane of the section; the small gaps might result from the two sub-units of the helix which would transcend the plane of the flagellar "section".

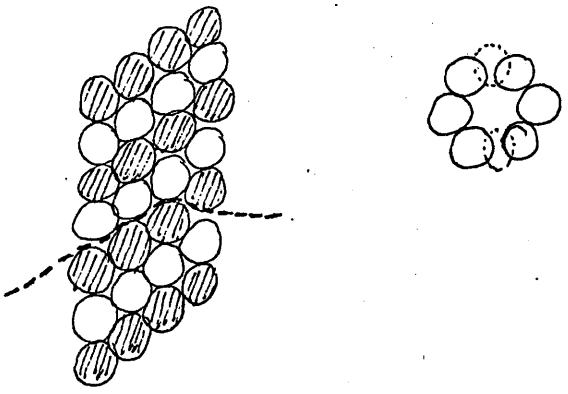


Fig. 35 Diagram to show a flagellum composed of two helically wound strands

However, this cross section could also be interpreted as evidence that there are in fact eight sub-units in a cross section of the flagellum and that the two helical strands run at a lower pitch angle than a model in which there are six sub-units in cross sections.

It has been noted that the angle the sub-units of S. typhimurium flagella make relative to the flagellar axis is greater than 45° (Lowy and Hanson, 1965). In the present study negatively stained preparations showed the flagella sub-units of P. vulgaris flagella to be at an angle of approximately 60° to the flagellar axis. If a model is constructed of one helically wound strand, with six sub-units in cross section, the sub-units are at an angle of under 60° to the flagellar axis; a model constructed of two helically wound strands, also showing six sub-units in cross section, gives a corresponding angle of just over 60°. In models constructed of one or two helically wound strands, with eight sub-units in cross section, the pitches of the sub-units from the flagellar axis are just under and just over 60° respectively.

It may be argued that the presumed flagella cross sections seen in the present study are artefacts and may, for example, represent an aggregation of stain around a central body. Alternatively they may be of biological but not of flagellar origin; for example the tail plates of bacteriophage. Bradley (1967) gives electron micrographs showing the tail plates of many bacteriophage. Examination of these micrographs shows that several have a tail plate which presents six sub-units but that these sub-units which constitute the tail plate are not spherical, in contrast to the sub-units of the presumed cross sections seen in the present study. Hence it seems unlikely that these presumed flagella cross sections are bacteriophage tail plates.

Images of the two sides of the flagellum can be obtained separately by the method of optical filtering and reconstruction described by Klug and DeRosier (1966) and DeRosier and Klug (1972). Sometimes in electron micrographs the two sides of a flagellum are clearly seen due to a "flattening" of the flagellum. If the sub-units of Proteus flagella

are arranged as indicated by the "cross sections" obtained in the present study, it would be possible under these conditions to see up to five sub-units across the width of the flagellum.

Polymerisation of flagellin solutions took place in the presence of high concentrations of salts. This polymerisation was accompanied by a marked increase in the viscosity and turbidity of the solutions and is in agreement with Lowy and McDonough (1964). When examined in the electron microscope, the reformed filaments resembled in vivo intact flagella in both their diameters and surface appearance. However, the filaments did not have the typical waveform of in vivo flagella but consisted of short straight lengths. Occasionally the reformed flagella filaments took the form of a mass of straight rods radiating from a central region. These structures were reminiscent of crystal clusters.

As noted earlier, Asakura, Eguchi and Iino (1964; 1966; 1968) have reported a process characteristic of crystallisation in which it is necessary to seed a flagellin solution before it will polymerise; the reformed flagella resemble those formed in vivo in their appearance, diameter and waveform. Perhaps Proteus flagellin solutions seeded with sonicated flagella fragments would have polymerised to give reaggregated flagella possessing the normal waveform? However, Abram and Koffler (1964) have reconstituted monomeric flagellin of B. pumilus at various pH values without the addition of seed.

If proteins have the same shape or conformation as the marker proteins used to calibrate a column of porous cross linked gel, the elution position of native proteins can be correlated directly with molecular weight. Andrews (1964, 1965) has shown that the elution volumes of proteins applied to Sephadex columns run in Tris HCl buffer correlate with their known molecular weights. When these elution positions are plotted against the logarithm of the known molecular weights of the proteins a linear relationship is obtained in the molecular weight range of 5,000 to 60,000.

The elution volumes of marker proteins from a column of Sephadex G 100 run in the Tris HCl buffer system of Andrews plotted against the logarithm of molecular weight gave a sigmoidal curve with a central linear region in the molecular weight range of 20,000 to 70,000. Andrews, as noted, gives a larger range for the linear portion of the curve but Fish, Mann and Tanford (1969) using columns run in the denaturing solvent guanidine hydrochloride have, on a similar plot, a linear region which falls between the molecular weights 20,000 and 70,000. This denaturing solvent, guanidine hydrochloride, dissociates each protein to its constituent polypeptide chains which behave in the solvent as linear random coils.

When monomeric flagellin was applied to Sephadex columns run in the buffer systems of Andrews (1964) the elution profile was extremely erratic. The absorbance of the eluate at 215 nm bore no relation to any of the marker proteins applied with the flagellin. However, cytochrome c was eluted in the expected position, as indicated by the absorbance of the eluate at 412 nm. It is likely that the marker proteins were eluted in their expected positions but were masked by the elution of the flagellin. Flagellin, dissociated before application to the column, was eluted over a very wide range and in a very erratic manner.

These studies to determine the molecular weight of the purified flagellin provide evidence on the possible conformation of the flagellin monomer. Prior to Bode, Engel and Winklmair's suggestion (1972) that the flagellin monomers were ellipsoidal, the present studies using column chromatography had already indicated that the flagellin monomers were of this form. Ellipsoidal molecules might become tangled and excluded from the pores of the gel (and thus eluted in the void volume) or "stuck" in the interstices of the filtration medium and be freed at various time intervals. This would account for the erratic elution profile of flagellin. In addition McDonough (1965) has calculated the polar ratio for Salmonella flagellins to be about 1.5. Fisher (1964) has suggested that this ratio, that is the ratio of the summed volumes of polar residues to non-polar residues, is a function of the volume and shape of the molecule. A polar ratio of about 1.0 would be expected if a flagellin molecule adopted a spherical configuration. In order to satisfy the surface area and volume requirements predicted by Fisher's hypothesis for a polar ratio of 1.5 the molecule would have to have a diameter of about 30 Å and a length of about 84 Å. Guanidine hydrochloride of higher molarity might convert the ellipsoidal flagellin monomers into linear random coils; in fact Joys and Rankis (1972) have recently used 6M guanidine hydrochloride to determine the molecular weight of S. paratyphi B SL 877 flagellin.

Due to failure in obtaining estimates of the molecular weight of flagellins by column chromatography both in Tris HCl and in molar guanidine hydrochloride buffer systems, the molecular weights of proteins and flagellins were assessed by measuring their mobilities on electrophoresis in polyacrylamide gels containing SDS. A constant binding ratio of SDS (1.4g SDS/g polypeptide) established for a range of proteins by Reynolds and Tanford (1970) was presumed to extend to flagellin. Hence the charge/mass ratio must be constant, the electrophoretic charge being

determined by the double layer on the outside of the particle; that is the coat of SDS. The relative migration of marker proteins in phosphate or Tris HCl gels containing SDS plotted against the logarithm of their molecular weights gave a sigmoidal curve, with a central linear region in the molecular weight range 20,000 to 50,000. Weber and Osborn (1969) state that the relationship is linear over at least the range 11,000 to 70,000; more recently other investigators have noted that the relationship is only approximately linear and that plots of logarithm of molecular weight against relative mobility are sigmoidal in nature (Neville, 1971; Williams and Gratzer, 1971). Williams and Gratzer note that the linear relationship between molecular weight and electrophoretic mobility breaks down for proteins with molecular weights less than about 15,000; below molecular weights of about 6,000 all proteins migrate with the same mobility. They state that the breakdown of this linear relationship is essentially independent of acrylamide concentration and interpret it in terms of the loss of asymmetry of the protein/SDS complexes in this range as predicted from the results of Reynolds and Tanford (1970). McDonagh et al (1971) state that there is a minimum molecular weight range that depends on the pore size of each gel. Molecules which are below this range have a faster electrophoretic migration than expected. For example, in their study they found that cytochrome c migrated faster than anticipated for its molecular weight in 7% acrylamide gels but had the expected mobility in 10% gels. However, flagellin does not fall into these low molecular weight ranges and the molecular weights obtained using this method (all in the range of 40,000) seem a reasonable estimate of the molecular weight.

Chang, Brown and Glazer (1969) have performed sedimentation equilibrium studies to assess the molecular weights of P. vulgaris Purdue strain flagellin; they obtained a value of 39,000 $\pm$ 1,600.

This value, and the values obtained for selected Proteus flagellins in the present study, are in distinct contrast to the molecular weight of 20,000 earlier reported for P. vulgaris Purdue strain flagellin by Erlander, Koffler and Foster (1960). These more recent results show that Proteus flagellins do not differ appreciably from other bacterial flagellins in their molecular weights.

It is of interest that Joys and Rankis (1972) report a molecular weight of 49,000 for the flagellin of S. paratyphi B SL 877. This value was obtained both by gel filtration in 6M guanidine hydrochloride incorporating 0.1M mercaptoethanol and by electrophoresis in polyacrylamide gels containing SDS. In the present study the one determination made of the molecular weight of S. typhimurium 1,2 flagellin assessed by electrophoresis in polyacrylamide gels containing SDS gave a value of 49,500. Like the results of Joys and Rankis (1972) this value is not in agreement with earlier published data. Analytical centrifugation of phase 2 flagellin of S. typhimurium indicates a molecular weight of about 40,000 (McDonough, 1965; Kerridge, Horne and Glauert, 1962; Asakura, Eguchi and Iino, 1964). Analysis of S. adelaide flagellin by analytical ultracentrifugation (Ada, Nossal, Pye and Abbott, 1964) gives a similar value of about 40,000. Similarly, Parish and Marchalonis (1970), utilising electrophoresis in highly dissociating conditions (pH 4.0; 9M urea) and a range of acrylamide concentrations, obtained a value of 41,000 for the same flagellin. In addition, under these conditions flagellin isolated from an unspecified strain of P. vulgaris yielded a molecular weight of 40,000.

As an aid to species identification, electrophoresis of proteins in starch gels and acrylamide gels has been used by many investigators. Robinson (1966) applied starch gel electrophoresis to a study of the esterases, catalases and peroxidases present in cell extracts of 24 strains of Corynebacterium. He found some strains differed in the relative migration of the various enzymes and in some instances more than one electrophoretic form was present for any one enzyme. He produced evidence that esterase analysis was of value in the separation of strains or serotypes, that peroxidases could be used as indicators of plant association and that catalases may be used for the separation of taxonomic groups above the species level.

Many more instances are known in which gel electrophoresis of selected proteins has aided the identification and classification of certain organisms. However, the relative migration of sample flagellins on electrophoresis in starch gels differed greatly both within and between flagellins isolated from different Proteus species. When the results were analysed statistically an F value of 1.8457 was obtained. This was not significant at the 5% level. Hence it is not possible with the present state of knowledge to use this method as a taxonomic tool in the classification of the genus Proteus. Slight changes in amino acid composition, commonly found between flagellins, can greatly affect the charge and consequent mobility of these proteins. In addition the procedure yields no information on the extent of the variation found. Certain amino acid substitutions could cancel themselves out; for example the substitution of an acidic amino acid for another acidic amino acid residue. However, since the method is so sensitive to changes in amino acid composition it may prove very useful for distinguishing between mutants of Proteus strains.

During the procedure used to obtain purified flagellar protein the ease with which the overall charge of the flagellin molecule could be altered without any change in amino acid sequence was demonstrated. As noted, great care was necessary when flagellar protein was purified by low pH treatment. Lowering the pH to 2 was far too drastic since a whole series of faster running bands appeared when these "purified" flagellins were electrophoresed in pH 8.65 starch gels containing urea. However single bands, corresponding in electrophoretic position to those of flagellins purified from flagella pellets, were obtained if the pH was only taken to pH 3. This change in the overall charge of the flagellin monomer is probably due to partial deamidation of the protein as a result of low pH treatment. Such proteins will have a greater net negative charge, the size of which will depend on the extent of the deamidation. Hence the objection of Martinez (1963a), that purification that includes pH 2 treatment does not yield homogeneous flagellar protein, still stands. However, purified homogeneous protein, capable of reaggregation, is obtained when the pH is only lowered to pH 3.

The amino acid analyses made for the various Proteus flagellins examined broadly agree with those published for other flagellins, and for Proteus flagellins (Chang, Brown and Glazer, 1969; Glossman and Bode, 1972). Histidine, not previously reported in Proteus flagellins, was present in many of the flagellins isolated from strains of this genus; cysteine was confirmed to be absent. It was thought that Proteus flagellins lacking histidine might be similar to the g... antigen series of Salmonella which lack histidine and have a phenylalanine to tyrosine ratio of 1.4 : 1. This ratio is approximately the reverse of the phenylalanine to tyrosine ratio for the other, histidine containing,

Salmonella flagellins. However, all the Proteus flagellins examined had a phenylalanine to tyrosine ratio of nearly 3 : 1. It is very possible that the amount of tyrosine was underestimated; there may have been some breakdown of tyrosine during acid hydrolysis and, in addition, the final amino acid peaks eluted from the 150 cm column of the amino acid analyser tended to broaden and were difficult to measure accurately. Hence the ratio for Proteus flagellins may not be as high as indicated.

The values for arginine found for P. vulgaris NCTC 100 20 and P. mirabilis NCTC 6197 flagellins were in general agreement with analyses published for flagellins isolated from members of the same species. P. morganii NCTC 232 and P. rettgerii NCTC 7475 flagellins each had slightly higher values for the number of arginine residues per molecule. ε NML has been reported in Salmonella (Ambler and Rees, 1959) and in Spirillum serpens flagellin (Glazer, DeLange and Martinez, 1969). This amino acid has been reported as possibly present in one Proteus flagellin; obtained from an unspecified strain of P. mirabilis (Glossman and Bode, 1972).

The presence of ε NML in certain Proteus flagellins (as the monomethyl form) was confirmed and the distribution of the amino acid studied throughout the genus. In the present study ε NML was confined to flagellins isolated from certain P. morganii strains; the amino acid was not found in all the P. morganii strains examined. The ratio of ε NML/lys (0.3) was appreciably smaller than that recorded for Salmonella flagellins (1.0) (McDonough, 1965). However, the Spirillum serpens flagellin studied had only a few ε NML residue per flagellin molecule (Glazer, DeLange and Martinez, 1969). It is of interest that the percentage of guanine plus cytosine in the P. vulgaris, P. mirabilis and P. rettgerii strains examined to date (Hill, 1966)

is about 40% while the ratio for the P. morganii strains examined is about 52%. The corresponding ratio for Salmonella species is about 52%.

The function of the methylation of the lysine residues can only be guessed. It has been suggested that methylation of lysine residues may reduce digestion of certain extracellular proteins of members of the Enterobacteriaceae by proteolytic enzymes present in the mammalian gut. Trypsin cleaves protein chains on the carboxyl side of lysine and arginine residues and it has been speculated that methylation of lysine may interfere with the cleavage of this amino acid. Benoiton and Deneault (1966) have reported that methylation of peptides decreases the susceptibility of the peptide to tryptic action. Interestingly, fragment A of S. adelaide flagellin, which contains 10 of the 11 ϵ NML residues located in the flagellin molecule, is exposed in the polymer (Parish and Ada, 1969). However Paik and Kim (1972) report little difference in the susceptibility of lys or ϵNML residues to cleavage by proteolytic enzymes, including trypsin. Hence the situation is not resolved.

In Salmonella a gene determining the presence or absence of ϵ NML in flagellar protein has been located (Stocker, McDonough and Ambler, 1961). These workers have suggested that methylation occurs after flagellin synthesis but this has not been definitely proved. The situation in the genus Proteus is unknown; as noted ϵ NML has not conclusively been reported in this genus before.

Comparative peptide maps each had a distinctive pattern although several peptides can be suggested to be in common with two or more maps. Since trypsin splits a protein on the carboxyl side of lysine or arginine

residues, the number of peptides should be one more than the number of lysine plus arginine residues. The expected and approximate number of peptides found are in reasonable agreement but the numbers of peptides found tend to be slightly more than the actual numbers expected. On most maps several peptides are not regularly found.

Specific reagents to identify amino acids showed the number of arginine, tyrosine and histidine containing peptides. The number of peptides containing specific amino acids were broadly in agreement with those expected from the amino acid analysis figures.

When peptide maps were made of tryptic digests of Proteus flagellins and compared, generally about a dozen spots were similar in their electrophoretic and chromatographic properties. However, even similarly placed spots showed slight differences in their mobilities. When digests of P. vulgaris NCTC 100 20 and P. mirabilis NCTC 6197 flagellins were mixed and mapped, at least 64 different peptide spots were detectable; some of the spots, however, were separated by very small distances.

It seems that peptide maps are extremely useful for characterising mutants closely resembling given flagellins (Yamaguchi and Iino, 1970) but not necessarily for characterising those flagellins which differ to a greater extent. Useful information is given regarding general affinities but the resolving power is possibly too great for determining the general relationships between members of the genus.

Even within a species, however, comparison of tryptic maps of P. vulgaris NCTC 100 20 and P. vulgaris NCTC 4175 reveals considerable differences between the peptide patterns. These differences are probably as great as those between peptide maps of flagellins isolated from different species of Proteus. Perhaps many of the similar regions of, for example, P. vulgaris NCTC 100 20 flagellin and P. vulgaris NCTC 4175

flagellin are contained within the insoluble core material formed if the protein is not maleylated or citraconylated prior to tryptic digestion. This argument could also be extended to flagellins isolated from different species and even genera.

Parish and Ada (1969) and Parish, Wistar and Ada (1969) have shown that of the four polypeptide fragments, A, B, CD and D, obtained by cyanogen bromide cleavage of S. adelaide flagellin in formic acid, the largest of the polypeptides (fragment A; molecular weight 18,000) contains all the antigenic specificities present on the flagellin recognised by the antisera used. Presumably the A fragment contains the bulk of the variant amino acid sequences present in at least certain Salmonella flagellins. McDonough (1965) has concluded that the antigenic specificity of flagellins resides in the sequence of the amino acid residues which will, of course, determine the conformation of the molecule.

There is considerable evidence that antigenic determinants are very limited in size as compared with a typical macromolecule, being equivalent in volume to, perhaps 4 or 5 amino acid residues. Modification of proteins, for example the substitution of ϵNH_2 groups of lysine residues by 2,4 dinitrophenyl, can give rise to considerable antigenic changes.

The N terminal amino acid for all the flagellins examined was alanine; the same as reported previously for the Purdue strain of P. vulgaris (Chang, Brown and Glazer, 1969) and S. adelaide (Parish and Ada, 1969).

The C terminal amino acid, arginine, is also similar to that of the flagellins isolated from various Salmonella strains (Parish and Ada, 1969; Davidson, 1971; McDonough, unpublished results). Parish and Ada (1969) suggest that the C terminal region of fragment C of S. adelaide flagellin (antigen fg), which is also the C terminal portion of the flagellin molecule, has the following amino acid sequence:

.... leu - leu - leu - arg COOH

McDonough (unpublished) has isolated the C terminal peptides of certain antigenically distinct Salmonella flagellins (including fg) and found them to have the following amino acid sequence:

.... (arg/lys), ser - leu - leu - arg COOH

The C terminal peptide of the Proteus flagellins examined could not be identified by the techniques used in the present study. This is in contrast to the C terminal peptides of the Salmonella flagellins examined by McDonough which were relatively easy to purify, although obtainable in low yield. Carboxypeptidase B digestion of Proteus flagellins released arginine, leucine and serine in the proportions of 1:2:1. It seems very likely that the sequence of the first few residues from the C terminal end is similar in Proteus and Salmonella flagellins. However after this point the sequences presumably diverge. If the C terminal peptide of the Proteus flagellins examined is contained within the "core material" there are presumably differences in the sequence or modification of the amino acid residues close to the C terminal end which account for this portion of the molecule being amongst the insoluble material formed on tryptic digestion relative to Salmonella flagellins.

Recently Joys and Rankis (1972) have isolated 29 tryptic peptides from S. paratyphi B SL 877 flagellin and determined their amino acid sequences. These tryptic peptides isolated account for 46.2% of the total amino acid residues present in the flagellin. Other tryptic peptides yielded insoluble aggregates which have, as yet, proved refractile to further study. Examination by these workers of the amino acid sequence of the purified peptides revealed in four of the peptides the presence of adjacent glycine residues. Wu and Kabat (1970) have suggested that regions of unusual flexibility, termed pivot positions, exist in the light and heavy chains of human and mouse immunoglobulin and consist of adjacent glycine residues or two glycine residues separated by a glutamine, a proline or an arginine residue. A possible parallel is provided in the results of Joys and Rankis.

The peptide amino acid sequences obtained in the present study represent the tentative sequences suggested for some of the tryptic peptides of P. vulgaris NCTC 100 20 flagellin.

None of the tryptic peptide sequences given closely resemble any of the tryptic peptides sequenced by Joys and Rankis. The ser - arg peptide (Number 26) isolated by these workers is also present in S. typhimurium flagellins (McDonough, unpublished results). In P. vulgaris NCTC 100 20 flagellin a ser - lys tryptic peptide (Number 21) occupies a similar position on peptide maps to the ser - arg tryptic peptide found in tryptic digests of S. typhimurium flagellins. No adjacent gly-gly residues were found amongst the few tryptic peptides sequenced of P. vulgaris NCTC 100 20 flagellin.

Until recently, tryptic, chymotryptic and peptic peptides coupled with large breakdown products obtained by non-enzymic reactions have been used to determine the amino acid sequences of various proteins. With the advent of a protein sequenator it is now possible to analyse a relatively long portion of the amino acid sequence of a protein. For example Crawford and Yanofsky (1971) have used an amino acid sequenator, together with gas liquid and thin layer chromatography, to determine the first fifty residues of the α chain of the tryptophan synthetase of a strain of Ps. putida. However, in most cases it is still necessary to obtain manageable fragments of the protein before the complete sequence of a protein can be deduced. To allow this certain factors need consideration, for example in the case of cyanogen bromide cleavage the number of methionine residues. In the present study it was theoretically possible to obtain three or eight cyanogen bromide fragments from P. vulgaris NCTC 100 20 and P. rettgerii NCTC 7475 flagellins; these have two and seven methionine residues respectively.

Very often when proteins are cleaved in various ways a relatively large proportion is obtained in an insoluble form. Various techniques used in the present study were found to be of use in keeping fragments of Proteus flagellar protein in solution. Maleylation or citraconylation, which place a substantial net negative charge on a protein, were both extremely effective in solubilising or keeping in solution flagellar protein fragments. For example, if citraconylated flagellar protein was digested with trypsin only a slight trace of insoluble material was found, in contrast to the usual heavy "core material" precipitate. Hence under these conditions it would be possible to sequence the "core material" peptides.

Citraconylation or maleylation blocks the lysine residues of a protein chain, hence the relatively large peptides obtained by cleavage of the molecule on the carboxyl side of the arginine residues can be sequenced to give overlapping regions of the total amino acid sequence.

In addition, it appears that citraconylation or maleylation will be invaluable in keeping even longer fragments of the flagellar protein in solution. Cyanogen bromide cleavage is very useful in protein degradation because of its high specificity in splitting methionine containing proteins into large peptides. However, the isolation, characterisation and sequence determination of big protein fragments may be very difficult because of poor solubility or aggregation under mild conditions, even in the cases of fully soluble globular proteins (for example; catalase, Schroeder, Shelton, Shelton, Robberson and Appell, 1969; S. adelaide flagellin, Parish and Ada, 1969). These difficulties can be circumvented by maleylating or citraconylating the cyanogen bromide peptides obtained before gel filtration and separation.

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