STRUCTURAL STUDIES ON FLAGELLINS FROM SHAPE AND ANTIGENIC VARIANTS OF *SALMONELLA* AND *PROTEUS* FLAGELLA.

by

Barbara S. Baker

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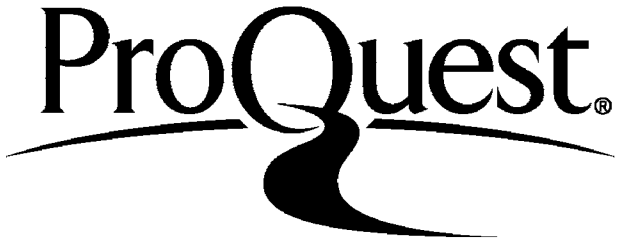
Department of Botany
Bedford College, London



ABSTRACT

The primary structures of flagellins from antigenic and shape variants of Salmonella and Proteus flagella were studied.

H antigenic differences in Salmonella are related to differences in amino acid composition of the flagellin molecules: peptide differences were observed between thermolytic, and between chymotryptic peptide maps of Salmonella g.... antigenic flagellins. These differences were located on the CNBr A fragments of the flagellins. Homologous CNBr A fragments were almost as effective as homologous flagella as inhibitors of antiserum raised against g.... antigenic flagella, as measured by immobilisation-inhibition and complement fixation-inhibition assays. Thus the H antigenic determinants appear to be located primarily on the CNBr A fragment, although some results suggest that they might extend onto the CNBr B fragment in certain flagellins.

The sites of amino acid substitutions in morphological mutants of Salmonella g.... antigenic flagellins appear to be located at either the N-terminal (CNBr B) or C-terminal (CNBr C and CNBr D) regions of the flagellin molecule. The C-terminus of the molecule is conserved.

Several ϵ-N-methyllysine residues were detected in most of the Salmonella g.... antigenic flagellins analysed and, in fewer numbers, in certain P. morganii flagellins. The absence of ϵ-N-methyllysine residues in flagellins of diverse bacteria was noted.

Arginine and tyrosine residues of various bacterial flagellins were differentiated by chemical modification into three groups: residues not accessible to modification; residues accessible in the monomer but not in the polymer; residues which are modified in both the monomer and polymer. Residues belonging to the second group may be implicated in the self-assembly of flagellin subunits into flagellar filaments.

Comparison of amino acid sequences of bacterial flagellins indicated sequences of homology at the N- and C-terminal regions of the flagellin molecule. These regions may be essential for the assembly of subunits into functional filaments. The central part of the molecule, however, appears to be a variable region which is exposed in the polymer and predominantly bears the H-antigenic characteristics of the bacterium.

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INTRODUCTION

HISTORY

The existence of flagella and their role as organelles of locomotion were first suggested by Ehrenberg in 1838. However, subsequently, Pijper put forward the theory that bacterial flagella were passive, useless appendages and were the result, not the cause, of cell locomotion (Pijper 1955; 1957).

This theory has been disputed by observations made at numerous laboratories and all investigators now accept the view that bacterial flagella are active organelles of locomotion. Various hypotheses have been proposed to explain the locomotion of flagellated bacteria. On the basis that flagella consist of two or three helical subfilaments, Jarosch (1964) suggested that helical waves are propagated along the flagellar helix as a result of small periodic changes in the pitches of the constituent subfilaments. Another hypothesis proposed by Doetsch (1966), and Vaituzis and Doetsch (1969) assumed that the helically shaped flagellum is rotated by a mechanism located around its base within the cell body.

A mechanism which avoids rotation of the flagellum relative to the cell body has also been proposed (Bütschli, 1883; Reichert, 1909). This latter hypothesis was favoured by Lowy and Spencer (1968) as being consistent with structural knowledge. However, more recent studies have accumulated a considerable body of evidence in favour of the theory of rigid rotation of flagella to explain the movement of bacteria (Berg, 1975a; b).

1. Structure of Flagella

a) General morphology

(i) Flagella filaments, hooks and basal bodies.

The bacterial flagellum consists of three morphologically distinct

parts: a helical filament external to the cell, a hook located at the base of the flagellum, and a basal structure closely associated with the cytoplasmic membrane and cell wall.

Flagella filaments

Bacterial flagellar filaments are long, uniformly thin and unbranched, and are attached at one end to the bacterial body via the hook structure. The filaments may or may not be sheathed. Stained filaments, seen under the light microscope or electron microscope, show in plan a sinuous form indicative of an *in vivo* helical form.

Using dark field microscopy, Mitani and Iino (1965) demonstrated this helical form in living specimens of *Salmonella abortivoequina*. Weibull (1950; 1960) observed the helical structure of flagella bundles using phase contrast microscopy, and showed that the helix is left-handed in strains of *Proteus vulgaris* and *Bacillus subtilis*.

The values for the sinusoidal wavelength obtained from flattened preparations examined under the light microscope (Leifson, 1960), or in the electron microscope by shadowing or negative contrast methods, agree reasonably well with the values for the pitch of the flagella helix measured in living material. The spiral width of the latter, however, is likely to be smaller than the amplitude of the flattened flagella.

Flagellar shape mutants of various bacterial strains have been isolated (reviewed by Leifson, 1960), the most commonly occurring mutant type being curly (Iino, 1962a). Curly flagella are characterised by a wavelength which is about half that of normal flagella, and a slightly smaller amplitude. Mutant bacteria possessing curly flagella have impaired motility. It has been reported that curly flagella are produced when phenylalanine is replaced by p-fluorophenylalanine in the culture medium of *Salmonella typhimurium* synthesising normal flagella (Kerridge, 1959).

Five different types of flagellar shape mutants have been isolated from a curly flagellar strain of Salmonella abortusequi: heteromorphous, small amplitude, para-curly, short and hooked-curly (Iino and Mitani, 1966). Straight flagella are occasionally seen among the normal flagella of many bacteria, but pure variants or mutants with straight flagella are rare (Leifson, 1960). Straight mutants were detected among non-motile mutants from normal strains of S. typhimurium (Iino and Mitani, 1967a) and Bacillus subtilis (Martinez et al., 1968).

Transduction analyses with P22 phage showed that the mutant sites responsible for flagellar shape are closely associated with a structural gene of flagellin, H1 or H2. Depending on which gene mutates, the mutant shape appears in phase 1 or phase 2 (Iino, 1962a; Iino and Mitani, 1967). Analysis of the tryptic peptide pattern of flagellin from a curly mutant of S. abortusequi revealed a difference in one peptide out of thirty five between normal and curly flagellin (Enomoto and Iino, 1966). A more detailed comparison, carried out on the flagellin of normal and straight flagella of B. subtilis identified the substitution of valine in normal flagellin for alanine in flagellin of the straight mutants (Martinez et al., 1968).

Reconstitution of flagellar filaments from monomeric flagellins, in vitro, also showed that the shape of the filaments was determined by the nature of the monomer (Asakura, Eguchi and Iino, 1966; Asakura, 1970).

In diphasic Salmonella strains, polymorphic alternation of flagellar shape is associated with phase variation (Iino, 1962a; Iino and Mitani, 1966; 1967a). Polymorphism has also been observed among the flagella on a single cell or among different regions of a single flagellum on certain bacterial strains (Hoeniger, 1965a; Iino and Mitani, 1966; Leifson, Carhart and Fulton, 1955). A single cell of Vibrio parahaemolyticus produces both polar and peritrichous flagella.

Interestingly, flagellar filaments of these two types differ from each other in both shape and antigenicity, suggesting that they are composed of different types of flagellin (Shinoda, et al., 1974; Miwatani et al., 1975). Thus polymorphism, as described above, appears to be caused by the production of flagellin molecules different in amino acid composition. However, the helical shape manifested by a flagellar filament is not always restricted to only one form; transformation of one form to another occurs often. This may be caused by the formation of flagellin molecules different in three-dimensional conformation, but with identical primary structures. For example, Hoeniger (1965b), and Leifson, Carhart and Fulton (1955) noted that normal-shaped flagella of Proteus species became curly-shaped when exposed to an acid environment. Reconstituted Salmonella flagella undergo reversible transformations at both acidic and alkaline pH's. Normal-shaped filaments are discontinuously transformed into coiled and curly-shaped filaments (Kamiya and Asakura 1976; 1977).

Curly flagella, formed by dialysis or long incubation of normal flagella, reverted to normal shape on the addition of ATP or pyrophosphate (Asakura, Eguchi and Iino, 1966; Oosawa et al., 1966).

Fixation by formalin causes a change of flagellar shape in some bacterial strains (Leifson, 1961). Normal or curly-shaped flagella are sometimes transformed into coiled, straight, small amplitude or typically undulant flagella.

The sudden change of a flagellar bundle from normal to curly, and vice-versa, observed under a dark-field microscope was termed "biplicity" (Pijper, 1955; 1957; Pijper, Neser and Abraham, 1956). Observations of flagellar bundles of various flagellar shape mutants of Salmonella showed that biplicity involves only specific flagellar types (Mitani and Iino, 1965; 1968; Iino and Mitani, 1966). This is also true

in flagellar fibres reconstituted by the copolymerisation of two different types of flagellins. For example, the copolymer of normal and straight flagellin shows one of three distinct shapes through a gradual change in the proportion of the two types of flagellin (Asakura and Iino, 1972).

Hooks

The hook, with its characteristic shape, connects the proximal end of the flagellar filament with the basal structure in the cell membrane. It can be distinguished from the filament not only by its morphology, but also by differences in fine structure, antigenic nature, and greater stability to a variety of agents.

In flagella of B. circulans, many of the hooks show a remarkable surface structure, giving the appearance of cross-banding (Abram, Vatter and Koffler, 1964). This surface structure has also been observed on hooks of flagella from B. stearothermophilus 2184. Further studies of this strain by Abram, Vatter and Koffler (1966) showed, just beyond the bend of the hook (which is smooth and 120-130Å in diameter), a region 300-350Å in diameter. This region consists of a central filament 100-200Å in diameter surrounded by a mat of fine fibrils (10-20Å in diameter), arranged in a right-handed helix around the central filament. The significance of this structure is not known.

Lowy (1965), studying the flagella of Pseudomonas rhodos, found that the proximal ends of sheathed and unsheathed flagella had a globular unit structure. Sheathed flagella with a lined fine structure had a sheathless region at one end which showed globular units and was about 200nm long, whereas in the corresponding sheathless flagella, a similar region was only 70nm long. In both cases the globular region had a bulbous appearance with a terminal taper. In unsheathed flagella with a globular fine structure however, the globular region was hook-shaped.

Raska, Mayer, Edelbluth and Schmitt (1976) observed two types of flagella, plain and complex, produced by a strain of Ps. rhodos. These flagella had correspondingly different hook structures. Plain hooks are cone-shaped, 70nm long and 13-21.5nm wide, consisting of helically arranged subunits, whereas complex hooks are cylindrical in shape, 180-190nm long, 15-16nm wide and are composed of globular subunits. The latter structure comprises four small-scale helical rows of subunits intersecting between 10 and 11 large-scale helices of pitch angle 80°. Also a core structure about 5nm wide is present inside the hook cylinder.

Flagellar hooks were first isolated free of filaments by Dimmitt and Simon (1971a) from B. subtilis using the fact that hooks are stable to heat, and filaments are not. The hooks consisted mainly of a protein with a 20% lower molecular weight than that of flagellin (flagellar protein) as shown by S.D.S. polyacrylamide gel electrophoresis. Antisera prepared against purified hooks was hook-specific. Earlier Lawn (1967) had shown that antibody to flagellar filaments would coat the filaments, but not the hooks.

Anti-hook serum to Salmonella SJ25 flagella prepared by Kagawa, Asakura and Iino (1973) reacted with hooks from a number of strains of Salmonella which differed from SJ25 in H and O antigens, flagellar shape and motility. Also, anti-SJ25 hook serum cross-reacted with hooks from E. coli W3110, but did not react at all with those from strains of Serratia, Proteus, Aerobacter and Klebsiella. The addition of purified anti-hook serum had little effect on motility, unlike the addition of anti-flagellar serum. Falkow, Rownd and Baron (1962) had also observed that E. coli had a common hook antigen with Salmonella.

Kagawa, Owaribe, Asakura and Takahashi (1976) determined the amino acid composition and molecular weight of hook protein from Salmonella SJ25 and obtained similar results to that for an E. coli "polyhook"

protein reported by Silverman and Simon (1972). Although hook protein from E. coli and Salmonella have the same electrophoretic mobility on S.D.S. polyacrylamide gels, Kagawa et al., (1979) showed that they had different isoelectric points; 4.7 for Salmonella and 4.4 for E. coli. The two hook proteins share common antigenic determinants as well as specific antigens, and their amino acid composition is more alike than that of their corresponding flagellins.

Very little is known about the function of the hook. It is thought to act as a "universal joint" at the base of the filament, and to allow the efficient transmission of rotational motion to the rest of the structure. The distal end of the hook structure acts as the initiation point for the growth of the filament (Iino, Suzuki and Yamaguchi, 1972), and the proximal end of the structure terminates in a rod inserted through the cell membranes to the basal structure (DePamphilis and Adler, 1971a; Dimmitt and Simon, 1971b).

Basal Bodies

The basal structure accounts for about 1% of the mass of the bacterial flagellum; however, it is the most complex part of the structure. This is probably a reflection of its proposed role, that of a reversible rotary motor. Bacteria swim by rotating their helical filaments; such rotation is driven by a rotary motor embedded in the cell membrane.

The existence of basal bodies at the base of flagella and cilia in protozoa prompted the early investigations of bacteria with the light microscope, and, subsequently, the electron microscope. Basal granules were recorded with the electron microscope for Chromobacterium violaceum and Vibrio metchnikovii (van Iterson, 1953), Rhodospirillum rubrum (Grace, 1954), Vibrio comma (Grace, 1954; Tawara, 1957) and for Vibrio and Spirillum species (Pease, 1956). However, all these observations

were made on shadowed preparations of cells that were either old or autolysed. Pijper (1957) and Kerridge (1961) therefore suggested that the basal bodies might be artefacts caused by cytoplasmic coagulation.

It has been known since the work of Weibull (1953) that the flagellum originates from the protoplast, and passes through the cell wall. Electron microscopy of sectioned cells (Murray and Birch-Andersen, 1963; van Iterson, Hoeniger and Nijman van Zanten, 1966a,b) did not clearly reveal its mode of internal attachment. Examination of partly lysed cells (or of flagella released from them) in shadow-cast or negatively stained preparations has proved more informative. Bacteria examined in this way include Vibrio metchnikovii (Glauert, Kerridge and Horne, 1963), Proteus species (Abram, Koffler and Vatter, 1965; Hoeniger, van Iterson and Nijman van Zanten, 1966a,b) and Bacillus species (Abram, Vatter and Koffler, 1966).

Glauert, Kerridge and Horne (1963), examining the sheathed flagellum of Vibrio metchnikovii, found that, although the sheath was continuous with the cell wall, the core of the flagellum terminated in a basal disc, 30-35nm wide, just inside the plasma membrane.

Abram, Vatter and Koffler (1964) reported that, in negatively stained preparations of Proteus vulgaris and in various strains of Bacillus, the flagella can be seen to arise via hooks from spherical structures, 25-35nm in diameter.

These findings were confirmed by Hoeniger, van Iterson and Nijman van Zanten (1966a,b) who showed, using potassium tellurite staining, that flagella of Proteus mirabilis terminated in rounded structures about 50nm wide, not in chondrioids, as was postulated. A brilliant dot occasionally observed in the rounded structures was interpreted as being part of the basal granule.

However, Abram, Koffler and Vatter (1965) in a more detailed study of basal structure and the attachment of flagella in cells of Proteus

vulgaris found the flagellum to arise from a spherical structure, 11-14nm in diameter. They postulated that the larger bodies, 20-70nm in diameter, consist, at least partly, of fragments of the cytoplasmic membrane folded around a smaller structure (the basal body proper). The latter appears to be equivalent to the brilliant dot seen occasionally in the larger bodies.

The detailed structure of the basal body was first revealed by Abram, Koffler and Vatter (1965). It consists of two pairs of discs separated by a thin connecting strand. Each pair of discs is approximately 20nm wide and 10nm long, the total length of the structure being approximately 30nm. One pair of discs is closely adherent to the base of the hook. These structures were also observed by Cohen-Bazire and London (1967) connected to the hooks of Rhodospirillum rubrum, R. molischianum and R. fulvum flagella. Vaituzis and Doetsch (1969) found the discs of polarly flagellated bacteria to be extremely resistant to lytic damage. However, the flagella prepared by Cohen-Bazire and London (1967) were contaminated with cell wall and membrane fragments. In addition, the enzyme used to lyse cells was a non-specific protease. Thus it was not until DePamphilis and Adler (1971a) succeeded in isolating "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, that basal body structure was finally clarified.

The basal body from E. coli is 27nm in length and consists of four rings, 22.5nm in diameter, arranged in two pairs and mounted on a rod. The top pair of rings are connected near their periphery, resembling a closed cylinder. In B. subtilis, the basal body is similar to that of E. coli except that the top pair of rings is missing. Further work by DePamphilis and Adler (1971b) showed the basal body ring closest to the hook (L ring) to be attached to the lipopolysaccharide membrane,

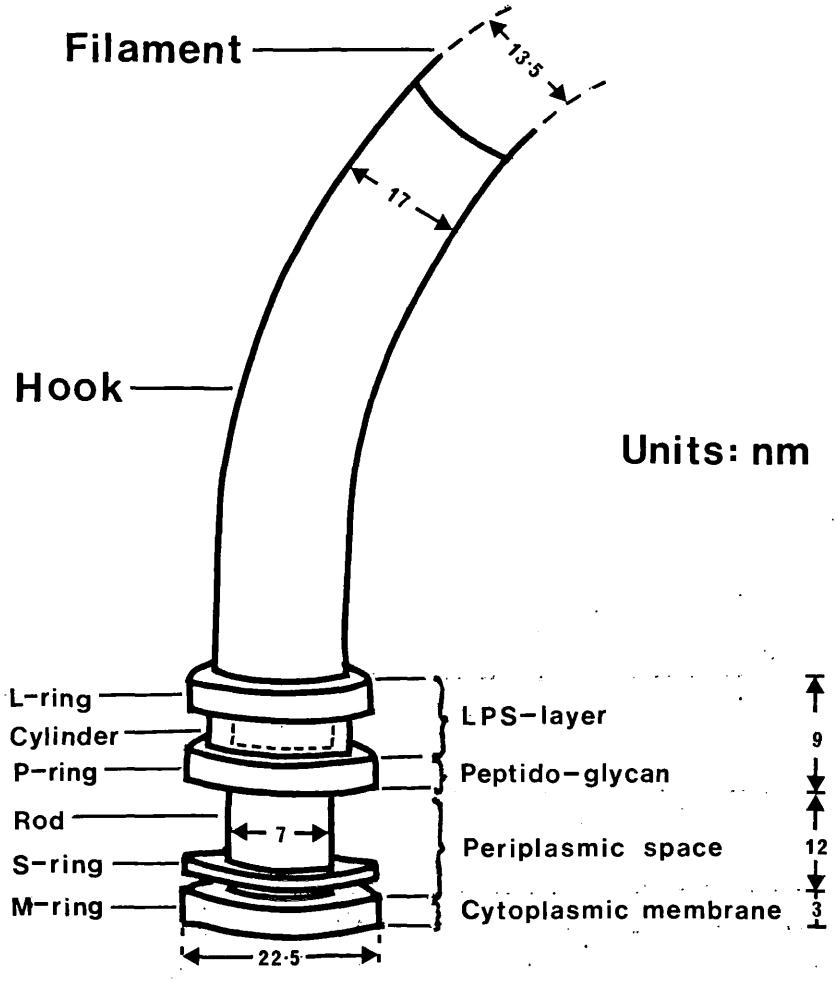


Figure 1: Model of the basal structure of a flagellum of S·typhimurium. (DePamphilis and Adler, 1971a)

and the ring furthest from the hook (M ring) to be attached to the cytoplasmic membrane. As the L ring is attached to the L membrane, and the total thickness of the L membrane and peptidoglycan layer is 12nm in E. coli, the second ring from the top would be in line with the peptidoglycan layer to which it is presumably attached (P ring) (Figure 1). There is no direct evidence for such an attachment.

The entire basal body structure can be separated from the rest of the filament by using relatively mild procedures designed to dissociate the filament. The surviving hook and basal body structure can then be separated and purified by differential centrifugation, isopycnic gradient centrifugation and finally by velocity gradient centrifugation (Hilmen, Silverman and Simon, 1974). Dissociation of these purified basal structures resulted in the resolution of 10 distinct polypeptide components (Hilmen and Simon, 1976). They include the hook subunit with an apparent molecular weight of 42,000 and 9 other polypeptides with molecular weights ranging from 60,000-9,000. The genes that correspond to these polypeptides have not yet been identified.

(ii) Chemotaxis: Response of bacteria to environmental changes.

Bacteria have a limited repertoire of movements which vary depending upon the size and shape of the cell and the distribution of the flagella. Most swim steadily in an almost straight line, then alter course abruptly. Motile E. coli swim by rotating their flagellar filaments in either a counterclockwise (CCW) direction which produces smooth swimming or a clockwise (CW) direction which results in tumbling movements (Larsen et al, 1974b). In wild-type strains the rotating flagella undergo frequent, random reversals (Larsen et al, 1974b; Silverman and Simon, 1975) and, as a result, the bacteria move in a

three-dimensional random walk composed of alternating smooth and tumbly episodes (Berg and Brown, 1972). As the organism swims, chemoreceptors (Adler, 1969) on the surface of the cytoplasmic membrane continuously monitor the concentrations of chemical in the immediate environment. Sudden large increases in attractant concentration (Larsen et al, 1974b) or decreases in repellent levels result in CCW rotation of the flagella, and thus smooth swimming. After several minutes, swimming behaviour and flagellar rotation return to their pre-stimulus patterns, indicating that bacteria possess a sensory system that undergoes adaptation. This adaptation mechanism allows bacteria to respond to concentration changes rather than to absolute concentrations of a chemical, and is therefore responsible for temporal detection of chemical gradients.

Thus control of flagellar rotation in response to chemical stimuli is the underlying basis of chemotactic behaviour in E. coli. The swimming pattern of the bacteria is assumed to reflect the level of some type of signal that controls the rotational machinery of the flagella. Thus whenever the signal rises above a threshold level, CCW rotation and smooth swimming result; whenever the signal falls below the threshold, CW rotation and tumbling result. Increases in attractant and decreases in repellent suppress tumbling; increases in repellent and decreases in attractant enhance tumbling. The conversion of stimulus information into a signal that modulates flagellar rotation is called stimulus transduction. Following stimulus transduction, a system for sensory adaptation restores tumble-controlling signals to the basal level.

Stimulus transduction

Wild-type E. coli is attracted to a number of sugars (Adler, Hazelbauer and Dahl, 1973) and amino acids (Mesibov and Adler, 1972),

and repelled by potentially harmful compounds such as ethanol (Tso and Adler, 1974). These chemicals are detected by specific chemoreceptors (Adler, 1969).

The galactose (Hazelbauer and Adler, 1971), maltose (Hazelbauer, 1975) and ribose (Aksamit and Koshland, 1974) receptors are water-soluble binding proteins which are located in the periplasmic space between the inner and outer membranes. Other sugars, including glucose, are detected by membrane-associated binding proteins (Adler and Epstein, 1974). These binding proteins are also involved in active transport of their respective ligands, thus binding protein mutants are also defective in sugar uptake (Hazelbauer and Adler, 1971; Hazelbauer, 1975; Aksamit and Koshland, 1974). Very little is known about the receptors for amino acids or repellents in E. coli. Competition experiments suggest that many different chemoreceptors are involved in mediating these responses.

Transmission of receptor information to the flagella seems to occur via a network of signalling elements. There are three known types of signalling mutants which have normal motility, but lack responses controlled by two or more receptors. The ribose-galactose signaller in E. coli may be the product of the trg locus. Mutants defective in trg function do not respond to ribose or galactose stimuli (Ordal and Adler, 1974). The tar (cheM) and tsr (cheD) loci are also involved in signalling, but at a later step than the trg product (Springer, Goy and Adler, 1977b). Loss of either function leads to numerous response defects. However, the Tar^- phenotype is complementary to the Tsr^- phenotype in that responses present in tar strains are absent in tsr strains, and vice versa. It is probable that all chemoreceptors may transmit signals through one or both of these components. Silverman and Simon (1977a, b) and Silverman et al. (1977)

constructed λ transducing phages that contained the tar or tsr region, and used them to program protein synthesis in various hosts. Both λ tar and λ tsr synthesised membrane proteins of approximately 60,000 molecular weight, dependent upon the presence of wild-type fla I gene in the host.

The signal involved in the excitation process has not yet been identified. However, very recent work in Adler's laboratory has suggested that the signal is a change in the intracellular level of a small molecule such as cyclic GMP.

Sensory Adaptation

During chemotaxis, both E. coli (Springer, Goy and Adler, 1977a) and S. typhimurium (Aswad and Koshland, 1974) have a continuous requirement for methionine. Methionine starvation eliminates tumbling in wild-type strains but fails to do so in a frequently tumbling mutant. (Aswad and Koshland, 1974). These results suggest that methionine metabolism is not tightly coupled to the generation of tumbles, but rather is necessary for the return of some tumble-regulating parameter to a steady state level. The main biochemical role of methionine in chemotaxis is that of a methyl donor in the form of S-adenosylmethionine (S.A.M.). Kort et al. (1975) identified a protein methylation reaction involved in chemotaxis of E. coli by examining cell proteins that incorporated radioactivity from labelled methionine in the absence of protein synthesis. The methyl-accepting chemotaxis protein (MCP) was located in the cytoplasmic membrane, and its behaviour on S.D.S. polyacrylamide gels closely resembled that of the tar and tsr proteins made by λ transducing phages. Direct comparisons on two-dimensional gels indicate that MCP probably represents the collective product of these two genes (Silverman and Simon, 1977c). Chemotactic stimuli alter the amount of methyl label associated with MCP (Silverman and

Simon, 1977c; Kort et al., 1975); increases in attractant stimulate methylation, and increases in repellent reduce methylation. These changes in the extent of MCP methylation are probably involved in the adaptation process (Springer, Goy and Adler, 1977a). Thus, adaptation to increases in attractant (tumble-suppressing) requires methionine and is correlated with methylation of MCP, whereas adaptation to decreases in attractant (tumble-enhancing) does not require methionine and coincides with demethylation of MCP. The tar and tsr products, therefore, seem to be involved in both stimulus transduction and sensory adaptation.

Mutants defective in che X function have a very low level of methylated MCP which might be responsible for the inability of che X strains to exhibit spontaneous tumbling (Kort et al., 1975; Minoshima and Hayashi, 1980). Furthermore, since adaptation to tumble-enhancing stimuli involves demethylation of MCP (Springer, Goy and Adler, 1977a), the very long response times of che X mutants may be due to the fact that most of the MCP is already in the demethylated state. Thus it seems likely that the rôle of the che X product is that of a methylase. A methylase activity has, in fact, been reported in S. typhimurium (Springer and Koshland, 1977), which appears to transfer methyl groups from SAM to membrane protein(s) that could be analogous to MCP in E. coli. The methylase is absent in smooth-swimming che R mutants of Salmonella (Springer and Koshland, 1977).

In addition to che X, che W also appears to be required for methylation of MCP.

A scheme showing the flow of information through the E. coli chemotaxis system is given in Figure 2. The input signal is eventually transmitted to the flagella, perhaps in the form of a specific ion flow. This signal could directly affect the flagellar rotor (Silverman and Simon, 1977c). There is some evidence that it may

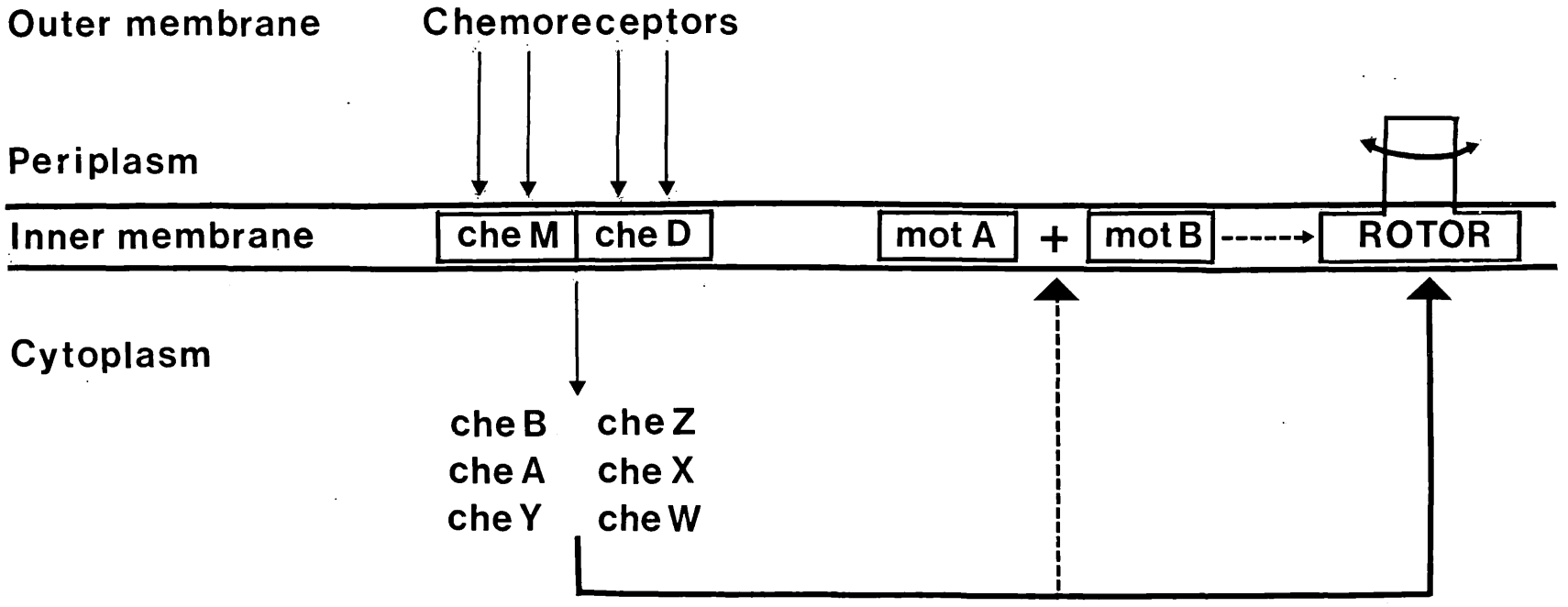


Figure 2 : The flow of information through the chemotaxis system

also affect the motility gene products. Szmelcman and Adler (1976) presented evidence for mot dependent changes in ion distribution upon the addition of chemoattractants or repellents.

(iii) Rotation of flagella

Various hypotheses have been put forward to explain the locomotion of flagellated bacteria. The Bütschli-Reichert hypothesis considered the flagellum as a cylindrical rod which had a hypothetical line of contraction running helically round its surface. When this line was shortened relative to other lines running parallel to it on the surface of the cylinder, the rod adopted a helical shape. Movement in a definite sequence of the line of contraction around the surface of the cylinder led to apparent rotation of the helically shaped flagellum, though it did not rotate relative to the body. Doetsch (1966), and Vaituzis and Doetsch (1969) favoured a theory of rigid rotation of flagella to explain the movement of bacteria. Since that time, Berg (1975a) has accumulated a considerable body of evidence in support of that theory. The most dramatic evidence has been obtained by tethering a cell with an abnormally long hook or a straight filament to a glass slide with anti-hook or anti-filament antibodies; the cell body rotates, alternately clockwise and counterclockwise (Silverman and Simon, 1975). The cell is non-motile when free, but spins several revolutions per second when the hook or filament is linked to the glass. Similar results have been obtained with anti-filament antibodies and cells with helical filaments (Larsen et al., 1974b). When polystyrene latex beads are linked to cells with straight filaments, the beads revolve about the axis of the filament in one direction, while the cell body rotates about this axis in the other.

Rotation of flagella would explain why cells with flagellar bundles are stopped by bivalent, but not by univalent anti-filament antibodies (Greenbury and Moore, 1966), whereas cells with only one

filament are inert to such bivalent antibodies (DiPierro and Doetsch, 1968).

Rotation of flagella would also explain how peritrichously flagellated cells are stopped by flagellotropic phages, (Meynell, 1961; Raimondo, Lundh and Martinez, 1968), how cells with straight filaments may be able to form flagellar bundles (Iino and Mitani, 1967a), and, finally, how cells with polyhooks, but no filaments, can counter-rotate when linked together with bivalent anti-hook antibodies (Silverman and Simon, 1972). Furthermore, as described in the previous section, it can be shown that chemotaxis is a function of the frequency of reversal of flagellar rotation.

Thus, it is now accepted that bacteria swim using flagella, helical filaments that are rotated by motors in the cell membrane. The motors are capable of rotating in either the counterclockwise (CCW) or the clockwise (CW) sense, and they alternate spontaneously between the two senses. In wild-type cells of Salmonella, E. coli and B. subtilis, the CCW rotational sense applied to the left-handed helical filaments provides thrust for swimming (Macnab, 1977), and brief CW intervals cause a random orientation or tumbling motion (Berg and Brown, 1972; Larsen et al., 1974b) by a quite complicated mechanism involving changes in flagellar structure (Macnab and Ornston, 1977).

Energetics of flagellar rotation

The motor utilises an intermediate in oxidative phosphorylation as an energy source, rather than ATP itself (Larsen et al., 1974a). The presence of ATPase has been detected in bacterial flagella (Barlow and Blum, 1952), but its very low activity suggests that it is a result of contamination. Recent studies on the mechanism of oxidative phosphorylation support the chemi-osmotic hypothesis of Mitchell (1961) in which the true nature of the intermediate form was supposed to be

the protonmotive force (PMF). A strong and direct support was obtained from the finding that an artificial PMF drove ATP synthesis in mitochondria, and also in bacterial cells (Wilson et al., 1976). According to this view, the PMF is expected to be the energy source for bacterial motility (Skulachev, 1977).

Manson et al. (1977) showed that the flagellar motors of Streptococcus strain V4051 can indeed be driven either by a transmembrane electrical potential (cell interior negative) or by a transmembrane pH gradient (cell interior alkaline). Unlike E. coli, S. typhimurium or B. subtilis, strain V4051 lacks an endogenous energy supply and stops swimming within minutes after being deprived of a carbon source. It also is highly responsive to ionophores and uncouplers. Starved cells spin in a mainly clockwise direction within ten seconds after the potassium ionophore valinomycin is added to induce a potassium diffusion potential (cell interior negative) or after the medium is acidified to generate a transmembrane pH gradient (cell interior alkaline). In either case, the angular velocity of the cells is a linear function of the protonmotive force (Manson, Tedesco and Berg, 1980). The angular velocity of metabolising cells is also inversely proportional to the viscosity of the medium. These results imply that the passage of a fixed number of protons carries the flagellar motor through one revolution, the threshold PMF for rotation appearing to lie close to OmV. The most remarkable finding made by the above workers was that starved cells also spin when protons move out of the cells in response to a diffusion potential or upon alkalinisation of the medium. Thus flagellar rotation can be driven by a protonmotive force of either sign.

Bacteria motility driven by electrical potential ($\Delta\psi$) across the membrane, or by proton chemical potential (Δ pH), has also been

demonstrated for metabolising cells of E. coli and Salmonella (Larsen et al., 1974a), B. subtilis (Shioi et al., 1978) and Rhodospirillum rubrum (Glagolev and Skulachev, 1978). At pH 7 to 7.5, pH values close to that of the cytoplasm, only a small proton chemical potential difference can exist across the membrane, so bacterial motility is driven by electric potential only. However ΔpH-driven motility has been less easy to demonstrate except in the case of B. subtilis which, unlike E. coli, regulates its total proton electrochemical potential (PMF) at a fairly constant value over a considerable pH range, with the result that at pH 5.5 it consists solely of ΔpH, and at pH 7.5 it consists (as it does for other bacteria) solely of $\Delta\psi$ (Khan and Macnab, 1980b). Measurements of motility of B. subtilis cells as a function of PMF at pH 7.5 and 5.5 indicated that a threshold of 30mV was needed for motility. A further increase to 60mV yielded a rapid increase in swimming speed, but the motors appeared to be saturated above 60mV, since no further increase in swimming speed occurred (Khan and Macnab, 1980b).

Protonmotive force not only drives motility, but was found to exert a regulatory effect on the switching of the motor between counterclockwise and clockwise senses (Khan and Macnab, 1980a). When motor speed was reduced by reducing PMF, the motor spent a higher fraction of time in CCW rotation, although the speed in both senses remained equal; at approximately 80% of the maximum motor speed under any given external load condition, no detectable CW rotation remained in most cells. This phenomenon has been demonstrated in Salmonella, E. coli and B. subtilis, and, in addition, in general chemotaxis mutants, including those known to be defective in the chemotactic methylation system. However, it is distinct from the tactic response to decreasing PMF in that it is permanent rather than transient, and

involves a development of CCW bias rather than of CW bias. Since responses to attractants and repellents do not involve an alteration in speed, Khan and Macnab (1980a) concluded that PMF force and chemotactic regulatory inputs to the motor operate in parallel.

Models of flagellar motor.

Any model of the bacterial flagellar motor must be compatible with the structure of the flagellar basal body (DePamphilis and Adler, 1971a,b), and to take into account its energisation by a PMF as described above. Most of the models proposed (Berg, 1974; Läuger, 1977; Glagolev and Skulachev, 1978; Macnab, 1979) can be made to meet these requirements. In a model proposed by Berg (1974), it was argued that the torque is generated between the M-ring, which rotates in the cytoplasmic membrane, and the S-ring, which is anchored to the rigid framework of the cell wall. The torque could be generated by the translocation, through the cytoplasmic membrane and the M-ring, of ions that interact with charges on the surface of the S-ring. This process could be catalysed by an asymmetric protein embedded in the cytoplasmic membrane at the periphery of the M-ring, with the direction of rotation determined by the nature of the asymmetry. Structures that might represent arrays of such proteins have been seen in freeze-fracture preparations of the gram-negative bacterium Aquaspirillum serpens (Coulton and Murray, 1978). The sites of insertion of the M- (or possibly S-) rings into the membrane are seen in convex fracture faces as circular depressions 23nm in diameter with raised central plugs about 10nm in diameter. Each depression is surrounded by a circlet of 15 small studs (sometimes 14 or 16). Manson, Tedesco and Berg (1980) suggested that these studs may be made up of a protein(s) similar to the mot A and mot B gene products of E. coli (Silverman and Simon, 1976; Silverman et al., 1976; Matsumura et al., 1977). The mot

polypeptides, which are required for flagellar rotation, are known to be located in the cytoplasmic membrane rather than in the flagellar basal body (Ridgway et al., 1977); however, their distribution within the membrane is not known.

b) Fine Structure of Flagella Filament

In shadowed preparations of several different bacterial flagella a large - scale helical structure has been observed. Starr and Williams (1952) described a triple helix with a pitch of about 500Å in a diphtheroid. Labaw and Mosley (1954) observed a double helix with a striation spacing of about 155Å in an unidentified organism, and (1955) a triple helix with a striation spacing about 190Å in a strain of Br. bronchiseptica. Marx and Heumann (1962) described a double helix with a 100Å striation spacing in Ps. rhodos; this was later identified as a sheath covering the flagella filament. The helical periodicities reported were much larger than those described by Kerridge et al. (1962) who observed hexagonally arranged globules about 4.5nm apart in negatively stained preparations of S. typhimurium flagella, partially degraded by ultrasonic vibration, by sodium dodecyl sulphate (SDS) or by heat treatment.

Early X-ray diffraction patterns of flagella from various organisms including S. typhimurium (Astbury et al., 1955; Beighton et al., 1958) show two notable features on the meridian: reflexions at about 5.1Å and 4.65Å indicating the presence of an α helical structure and of a cross-β structure, respectively; a periodicity at 410Å, which is also a feature of muscles. On the basis of these results, flagella were assumed by Astbury to be of the nature of monomolecular hairs or muscles.

In flagella of Ps. fluorescens, Ps. rhodos and Proteus vulgaris Lowy and Hanson (1964; 1965) observed two types of flagellar fine

structure, A (beaded) and B (lined). One of these types (A) has been found in S. typhimurium, and the other (B) in B. subtilis. In Ps. rhodos, a single flagellum can show both types of structure. The A structure shows helically connected globules aligned in longitudinal rows; sometimes there are also indications of longitudinal connections. The B structure shows neither globules nor helices, but has thick, longitudinal lines, the number of which in a given species is the same as the number of rows of globules in the A form.

(i) Salmonella flagella

On the basis of equatorial diffraction data, Burge (1961) proposed two alternative models for the flagellar structure found in the Salmonella type. One is made up of three α-helically wound strands, and the other is a cylinder of seven hexagonally-packed strands, (6 at the periphery of the cylinder and 1 inside) which could be either longitudinally orientated or α-helical.

Kerridge et al. (1962), having examined Salmonella flagella by electron microscopy, proposed two alternative models of flagellar structure, in each of which the globules are lined up in longitudinal rows and alternate with each other in adjacent rows. In the first model, the connections between the globules are longitudinal. In the second model, the globules are connected obliquely so as to form three helically wound strands. Kerridge et al. favoured five longitudinal rows for Salmonella mainly because the transverse sections of flagella show a regular pentagonal arrangement of globular units. Lowy and Hanson (1965) proposed a modified model to that of Kerridge et al. for Salmonella flagella in which eight longitudinal rows of helically connected flagellin molecules align in such a way that the number per turn of each helix equals the number of longitudinal rows, and the molecules form a cylinder. The outside diameter of the cylinder is

163Å, the effective diameter of the core inside the cylinder 60Å, and the longitudinal rows of units are 43Å apart. The proposal of eight longitudinal rows, rather than five as favoured by Kerridge et al., was based on the observation of four or five rows on the surface of negatively stained flagella. This model is more consistent with the early X-ray diffraction analyses (Astbury et al., 1955; Beighton et al., 1958) than the models of Burge (1961) or Kerridge et al. (1962).

Further X-ray diffraction studies (Champness and Lowy, 1968; Champness, 1971) of S. typhimurium show a meridional periodicity of 52Å, confirming observations made under the electron microscope (Lowy and Hanson, 1965). The X-ray patterns obtained were consistent with the general "staggered-row" configuration proposed in the models of Kerridge et al. (1962) and Lowy and Hanson (1965). However, measurements of the maximum on the first layer line show that the spacing between longitudinal rows is about 60Å, and not 43Å as predicted in Lowy and Hanson's model. The arrangement indicated by the X-ray pattern suggests that the units are ellipsoidal rather than spherical as indicated in Lowy and Hanson's model. No evidence for a meridional periodicity of 410Å, or the existence of a cross-β structure, both reported by Astbury et al. (1955), was obtained in these studies.

From low angle X-ray diffraction studies, Champness (1971) obtained a value of 130Å for the diameter of S. typhimurium flagella, as compared to values of 140Å and 180Å measured in the electron microscope after staining with phosphotungstic acid and uranyl acetate, respectively.

Burge and Draper (1971) studied the equatorial X-ray diffraction patterns of S. typhimurium which provide valuable information concerning the flagella diameter and the packing of subunits in the polypeptide chains. Their results suggest a value of 140Å for the

diameter of S. typhimurium flagella which is 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 the stain into a central core. The effect of hydration and of various stains on the equatorial X-ray diffraction patterns of flagella suggest that the interpretation of results needs great care.

Using the straight filaments of a mutant Salmonella isolated by Iino and Mitani (1967a), O'Brien and Bennett (1972) analysed the flagella structure in detail using optical diffraction and filtering methods. Straight filaments are more convenient for optical diffraction of electron micrographs and might be expected to form better orientated specimens for X-ray diffraction. However, better orientation was not obtained, but optical diffraction patterns of the straight flagella were better than those of normal flagella. Thus the straight flagellar structure can be regarded as a single genetic helix with just under eleven subunits in two turns (or 82 subunits in 15 turns). There are eleven long-pitch helical rows running at an angle of about 7° to the helix axis. This angle is in the range predicted by Asakura (1970) from theoretical calculations based on observations of low resolution electron micrographs of flagellar polymorphs of differing over-all helical waveform. In normal flagella these rows run at less than 2° to the length of the flagellum, and their helical pitch is about 2.5μm as compared to 0.4μm in straight flagella. A drawing of the structure with non-spherical subunits demonstrates the polarised appearance of the flagella and suggests a simple model for the V-shaped breaks sometimes observed in fragmented flagella. Highly elongated subunits were proposed on the basis of the hydrodynamic data, and the data obtained by small-angle X-ray scattering experiments (Bode et al., 1972).

A three-dimensional model of flagellin, reconstructed to an effective resolution of about 14Å by Shirakihara and Wakabayashi (1979), proposed three different candidates for the shape of flagellin. All of them are elongated and consist of at least four regions (C, I, M and S) per subunit. It is interesting that in the three-dimensional structure of muscle thin filaments reconstituted to a resolution of 15Å, actin molecules did not show such a beaded submolecular structure (Wakabayashi et al., 1975).

Optical diffraction and filtering studies carried out on straight flagella from a mutant E. coli (Kondoh and Yanagida, 1975) indicate a structure consisting of a basic helix with eleven subunits in two turns. A characteristic of it is that one class of the helical rows of subunits runs closely parallel to the filament axis. This structure is definitely different from that of the straight flagellar filament from a Salmonella mutant (O'Brien and Bennett, 1972).

The E. coli mutant of Kondoh and Yanagida, and the Salmonella mutant of O'Brien and Bennett, were suggested by Kamiya et al. (1979) to be examples of the two types of straight flagella predicted by current models of bacterial flagella.

Several models have been proposed to explain the polymorphic nature of homogenous polymers of a single kind of flagellin. Klug (1968) postulated that there are two sets of bonds at different radii with slightly different helical parameters, and that the pitch of the waveform was determined by the beat between the pitches of the two sets of bonds. Asakura (1970) proposed a model in which flagellin molecules were capable of adopting two stable conformations (R and T). In comparison with type R subunits (or strands), type T subunits (or strands) are slightly longer and, in addition, subject to a slight shear deformation. If all strands comprised in a flagellum are of type R or type T, its axis will become straight. If, however, a few

adjacent strands in the total strands are of type R and the other of type T, then the axial line will assume a large-scale helix.

Although certain features of Asakura's model are inconsistent with current knowledge of flagellar structure, the assumption of two discrete states in flagellin conformation has been used as a basis for subsequent models. In a mechanical model, Calladine (1976; 1978) proposed that flagellin molecules behave as bi-stable elastic blocks with alternative bonding sites either on the surface of or within the subunit. The model predicts a simple relationship between the number of longitudinal columns of units in the helical surface lattice and the number of helical forms which can be built. Two of these helical forms, the straight flagellum with an extreme right-handed twist (about 7° at the surface of the flagellum) and the straight flagellum with an extreme left-handed twist (2° to 3°) are represented by the E. coli and Salmonella mutants, respectively. Kamiya et al. (1979) proposed a model very similar to that of Calladine (1978) in which 12 types of polymorphs can be constructed, but specific shapes of subunits were not assumed.

(ii) Other bacterial flagella

Certain bacterial flagella, but not those of Salmonella, are characterised by the presence of strongly marked longitudinal lines. Ps. fluorescens flagella show 5 or 6 lines, the width of the flagellum being about 170Å (Lowy and Hanson, 1965). However, Ps. rhodos, P. vulgaris, B. pumilis and B. subtilis flagella show 4 or 5 lines, with a correspondingly smaller flagellar width. For example, a value of about 140Å for B. pumilis and B. subtilis (Lowy and Hanson, 1965).

A model has been constructed for the flagella of B. pumilis in which ovoid subunits are arranged in the form of six fibres coiled around an empty core (Abram, Vatter and Koffler, 1964; Abram, Koffler and Vatter, 1966a). X-ray diffraction patterns of flagella from

B. pumilis (Champness and Lowy, 1968) show that, as in the case of Salmonella, all the reflexions in the meridional direction can be indexed as orders of about 52Å. Intensity differences between patterns given by Salmonella and by B. pumilis, indicate a difference in structure of subunits, but geometrical arrangement of subunits is basically the same in all types of flagella. From this point of view, the main differences in the surface appearance of the two forms are that the helical connections between the subunits apparent as oblique rows are more emphasised in beaded flagella, whereas the interconnections between subunits apparent as longitudinal rows are more emphasised in lined flagella. However, just as longitudinal rows of subunits can be observed in beaded flagella, rows of units with an axial spacing of about 50Å are sometimes visible in lined flagella.

In Ps. rhodos, both beaded and lined surface structures were found. Many of the flagella with lined structures had sheaths, whereas those with globular structures did not. Furthermore, some flagella had the lined structure and were sheathed along part of their length, but ended in a region which lacked a sheath and showed the globular structure.

Different surface structures were found in different P. vulgaris strains. All the flagella in cultures of strain 4175 of P. vulgaris showed the globular structure with either 4 or 5 longitudinal rows of globules (Lowy and Hanson, 1965). However, in an unknown strain of P. vulgaris all the flagella showed the lined structure with either 4 lines seen more or less in face view, or 5 lines of which 2 appeared in profile. A few of the lined flagella had sheaths and were wider than those without one.

At frequent and fairly regular intervals (commonly 600-800Å) along many of the lined flagella of Ps. fluorescens, short regions (about 150-200Å long) are observed in which all the lines disappear. In some of these flagella, the lines on either side of each interruption appear

displaced by half a period relative to one another. In Ps. rhodos also, such a shift has occasionally been seen. However, there is a strong possibility that these features are artefacts; the helical shape of a flagellum is severely distorted when it is flattened onto a carbon film. This might result in the destruction of inter-molecular bonds and ordered structure at certain periodically distributed sites (the discontinuities), at which also 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).

(iii) Sheathed Flagella

In addition to bacterial flagella composed only of flagellin, Lowy and Spencer (1968) described another category of flagella which have a banded structure around on otherwise normal looking flagellum. The banded structure can consist of either close-fitting helically wound bands, or a loose-fitting sheath. Two close-fitting helically wound bands about 25Å thick have been reported in negatively stained preparations of Ps. rhodos (Lowy and Hanson, 1965). The pitch of each helix is about 254Å. If it is assumed that contrast is greater on the upper surface of the flagellum, the helices would be designated as left-handed. This agrees with the results of Marx and Heumann (1962) who observed two left-handed helices (pitch about 200Å) in shadowed preparations of Ps. rhodos. The fine structure of the flagellum can sometimes be seen through the sheath, and both aspects of the sheath helix are visible. Similar structures were also found by Lowy and Hanson in a strain of P. vulgaris, although no clear indications of helical structure were observed. An unusual strain of Ps. rhodos isolated by Schmitt et al. (1974) was found to produce two morphologically distinct flagella termed plain and complex. Fine structure analysis of the complex flagella revealed a helical sheath, 18nm wide, of 3 close-fitting helical bands, each about 4.7nm wide,

separated by 4.7nm axial intervals, running at an angle of 27°. The internal core has a similar, but not identical, substructure to plain filaments.

Loose-fitting sheaths are found round flagella of many bacterial strains and can often be seen isolated as a coherent structure. DeRobertis and Franchi (1952) reported a trypsin-digestible sheath surrounding the flagella of B. brevis. The flagellar sheath observed by van Iterson (1953) in Vibrio metchnikovii was later found to be a continuation of the cell wall (Glauert et al., 1963). Follett and Gordon (1963) removed the sheath in V. metchnikovii with urea or acid, or by autolysis leaving the flagellar filament intact. They concluded that the sheath probably differs in chemical composition to the flagellar filament and that it may be of cell wall origin. However, an unsheathed flagellar filament would normally be degraded under these conditions. The stability of the filament may be explained by the fixation of the flagella with formalin prior to examination.

The flagellar sheath protein of V. cholerae has recently been characterised by Hranitsky et al. (1980), and shown to be composed of 3 polypeptides with minimum molecular weights of 61,500, 60,000 and 56,500. Serological experiments suggested that the sheath was not an extension of the outer membrane of the cell.

Around the flagella of Spirochaeta stenostrepta, Holt and Canole-Parola (1968) reported the presence of a sheath showing alternating light and dark bands with a periodicity of about 3-4nm. Similar cross-striations were observed by Bladen and Hampp (1964) on the axial filaments of a small oral treponeme.

An examination of the flagellar filaments of different H serotypes of E. coli revealed a surprising degree of variability in their ultrastructure (Lawn et al., 1977). The flagellar filaments of some morphotypes have a surface structure which, in other genera of

enterobacteria, has been attributed to the presence of a separate sheath (Lowy and Hanson, 1965; Schmitt et al., 1974). However, an additional sheath protein was not revealed by S.D.S. polyacrylamide gel electrophoresis, and polymerisation experiments indicated that the assembly pattern of flagellin molecules is probably the same in all E. coli flagella (Lawn, 1977).

The above, and other evidence suggests that there is no true sheath, but that the differences in flagella surface structure between different E. coli flagella are the result of differences in the superficial parts of the flagellin molecule. This idea is compatible with a three-dimensional model of bacterial flagella presented by Shirakihara and Wakabayashi (1979).

(iv) Central Core?

The question as to whether or not the core of the flagellum is empty remains an unsolved problem. Although Kerridge et al. (1962) reported that, in thin sections, the flagellum of S. typhimurium appeared hollow, the presence of a core in negatively stained flagella can only be detected as a thin line of stain after rigorous physical treatment. For example, ultrasonic treatment of Acetobacter suboxydans flagella (Claus and Roth, 1964) and autolysis of V. metchnikovii flagella (Glauert et al., 1963) revealed the presence of a core of diameter 1.5nm and 1.2nm, respectively. Although Lowy and Hanson (1964) found no evidence for a core in untreated P. vulgaris flagella, Swanbeck and Forslind (1964) evaluated a cylindrically averaged radial density distribution for flagella detached from P. vulgaris, and consider that it supports the idea that flagella have a hollow central core. Burge and Draper's (1971) detailed study of the effects of hydration and staining on flagella specimens supports this theory. Lowy and McDonough (in Lowy et al., 1966) observed the appearance of a large number of finer, fibrous structures on treatment of synthetic

flagella-like filaments with trypsin. These fibres had an overall width of 40-60Å, showed a twisted appearance, and along their length, two lines can often be discerned. It was suggested that these fibres may make up the core of the flagellum. Hotani et al. (1969) and Martinez et al. (1972) observed similar structures on digestion of isolated S. typhimurium flagella with trypsin. Martinez et al. succeeded in isolating these fibres as, unlike flagella, they are 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 antiserum. Chymotrypsin treatment of Salmonella flagella also produces fibres, but fibres are never seen in tryptic hydrolysates of flagellin monomer. Hotani et al. (1969) suggested that the fibres might represent an in situ association of large tryptic peptides, but the evidence does not exclude the possibility that a central core exists; the tryptic peptides might be attached onto some partially digested core.

c) Flagellin, the structural protein of flagella

(i) The chemical component of flagella

Isolation and purification of flagella

Isolation of bacterial flagella involves the mechanical breaking of flagella from cell bodies followed by their separation by centrifugation. Craigie (1931) in a study of the serological reactions of the flagella of B. typhosus, detached flagella from the cell bodies by forcing a heavy bacterial suspension through a vertical capillary jet. However, a shaking machine or a blender has been most often used for the breaking (Gard, 1945; Weibull, 1948; Kobayashi et al., 1959; Keeler et al., 1966). The conditions for breaking off flagella from bacterial cells with minimal destruction and without a decrease in the viability of the cells were investigated by Stocker and Campbell

(Stocker, 1957; Stocker and Campbell, 1959).

Purification of isolated flagella was achieved by repeated differential centrifugation (Gard, 1945; Weibull, 1948; Kobayashi et al., 1959). Purified P. vulgaris flagella was subjected by Weibull (1948) to chemical and physiochemical analysis. He found that bacterial flagella are reversibly precipitable with ammonium sulphate (Weibull, 1949a), a property he subsequently used to further purify flagella preparations of P. vulgaris and B. subtilis (Weibull, 1949b). Experiments involving stepwise ammonium sulphate saturation of flagellar suspensions by Koffler and Kobayashi (1957) indicated that, unlike flagella of some bacterial strains, non-flagella proteins and other contaminants can be precipitated selectively. Thus by discarding the least homogenous fractions, fairly pure preparations of flagella were obtained.

Kobayashi et al. (1959) found that purification of flagella by centrifugation is also aided by the removal of the "centre-bottom" portion of precipitates, a fraction that often contains impurities.

Elution of flagella from a diethyl aminoethyl (DEAE) cellulose column by NaCl gradients was used by Martinez (1963a) to further purify flagella from Spirillum serpens, B. subtilis and P. vulgaris.

Other methods of purification used involve low-temperature ethyl alcohol precipitation (Keeler et al., 1966) and preparative zone electrophoresis with a powdered co-polymer of polyvinyl chloride and polyvinyl acetate as supporting medium (Miwatani, Shinoda and Fujino, 1970). This latter method proved to be very effective in removing the spherical bodies that contaminate flagellar preparations of Vibrio parahaemolyticus.

Dimmitt and Simon (1971b) have purified relatively intact flagella from bacterial lysates of B. subtilis. Flagella prepared in this way maintain the hook structure, the presence of which increases the

thermal stability of the flagellar filament.

The protein nature of highly purified flagella has been demonstrated in various bacterial strains (Weibull, 1950; Kobayashi et al., 1959). However, there is a possibility that, in certain bacteria, minor non-protein entities, which are loosely bound to the native structure, are eliminated during purification. An exceptionally tight binding of non-protein substance to the flagellar protein was reported in the flagella of B. stearothermophilus (Abram and Koffler, 1964) and Spirillum serpens (Martinez, 1963b). In these bacteria carbohydrates are bound to flagellar fibres and are disaggregated only when the flagella are dissociated with acid. The release of lipid from purified flagella of unspecified source upon ether extraction was reported by Poglazov (1966).

Preparation of flagellin

Flagella can be dissociated by a variety of agents giving rise to the flagellar protein, flagellin, a term originally used by Astbury et al. (1955). Acid dissociation, at pH 3.0 is the most widely used method (Weibull 1948, 1949a,b; Koffler and Kobayashi, 1957) producing homogenous subunits of molecular weight approximately 40,000. Impure flagellar preparations leave a residue of "pH 2 - insoluble material" after centrifugation; this residue contains the bulk of the non-protein substances in the preparation (Kobayashi et al., 1959). Breakdown of P. vulgaris flagella in acid was found to follow pseudo first-order kinetics with regard to uptake of hydrogen ions (Vegotsky et al., 1965). Evidence from ultracentrifugation was in favour of a fully cooperative breakdown of flagella.

Other dissociating agents include alkali (Pijper, 1957), detergents, phenol, sonic oscillation (Koffler, Mallet and Adye, 1957), heat (Martinez and Roscenberg, 1964; Dimmitt and Simon, 1971a) and compounds capable of breaking hydrogen bonds, such as urea (Koffler,

Mallet and Adye, 1957; Shinoda et al., 1970).

In P. vulgaris, the dissociated flagellin exists as a monomer below pH 3.8 in the absence of salt, and as a dimer above pH 4.5; the dimer is stable up to a pH of about 12 (Erlander, Koffler and Foster, 1960). The change in optical rotation of a flagellin solution observed when its pH is lowered below 3.8 was interpreted as an unfolding of the monomer accompanied by considerable loss of secondary and tertiary structure. This phenomenon has not been observed for other flagella, thus its significance is somewhat questionable. In acidophilic Thiobacillus thiooxidans the polar monotrichous flagellum is operationally active at a hydrogen ion concentration measured as low as pH 0.6.

The homogeneity of flagellin molecules obtained by acid dissociation of a single kind of flagella from several different bacterial strains has been demonstrated by both physical and chemical analyses. A single peak in the analytical ultracentrifuge was obtained for P. vulgaris (Weibull, 1948; Erlander, Koffler and Foster, 1960), S. typhimurium phase 2 flagella (Kerridge et al., 1962) and S. adelaide (Ada et al., 1963, 1964), B. subtilis and S. serpens (Martinez, Brown and Glazer, 1967). Kobayashi et al. (1959) showed that, although flagella precipitate over a wide range of ammonium sulphate concentrations, flagellin comes down within a very narrow range of saturation concentrations. Chromatography of S. abortusequi flagellin (Enomoto and Iino 1962, 1966) and polyacrylamide gel electrophoresis of flagellins of B. subtilis and S. serpens (Martinez et al., 1967) and P. vulgaris (Chang, Brown and Glazer, 1969) gave one band in each case. However, use of the latter technique has demonstrated the need for prior purification and controlled conditions during acid disaggregation of flagella. Studies with B. subtilis (Frankel, Martinez and Simon, cited in Joys, 1968) have shown that purification of flagella by

differential centrifugation followed by acid disaggregation in the presence of salts, yield preparations which show multiple bands on polyacrylamide gel electrophoresis. However, additional purification of the flagella by chromatography on a DEAE cellulose column prior to disaggregation, and treatment with acid in the absence of salts resulted in a preparation yielding only one band (Martinez, Brown and Glazer, 1967).

A highly homogenous solution of flagellin monomers was obtained by thermal dissociation of Salmonella flagella (Asakura, Eguchi and Iino, 1964). Kinetic studies of the thermal transition of flagella to flagellin in S. serpens was studied by Martinez and Roscenberg (1964). They found that the thermal transition exhibited by flagella closely follows the process of transition reported for many soluble proteins (Hermans and Scheraga, 1961), and is similarly affected by low ionic strength, pH and urea. The effective temperature for the dissociation of flagella differs in different bacteria. Generally, flagella of thermophilic bacteria are more resistant to high temperature than those of mesophilic bacteria, the difference being attributed to molecular differences in the flagellin of mesophiles and thermophiles, rather than to extraneous factors (Koffler, Mallet and Adye, 1957; Koffler, 1957).

There are examples, apart from that of phase variation in Salmonella, of more than one type of flagellin being synthesised. Sullivan et al (1969) reported that B. pumilis synthesised two very similar flagellins which could be separated by ion-exchange chromatography or polyacrylamide gel electrophoresis. Shinoda, Miwatani and Fujino (1970) reported that flagella from Vibrio parahaemolyticus were comprised of two different subunits. Flagella, purified by preparative electrophoresis on Pevikon c-870 and solubilised by treatment with 6M urea, were fractionated into the two

subunits (U-I and U-II) by hydroxylapatite column chromatography. The subunits both had a molecular weight of about 40,000 and had similar physicochemical properties. However, they differed in their antigenicities and amino acid compositions. Subsequently, Shinoda et al., (1974) reported that the subunits U-I and U-II corresponded to the flagellins of peritrichous and polar monotrichous flagella of V. parahaemolyticus, respectively. The flagellin of V. alginolyticus has also been isolated by Miwatani and Shinoda (1971) and shown to be similar in its immunochemical and physicochemical properties to that of the subunit U-II in V. parahaemolyticus flagellin.

More recently, Fukuda et al. (1978) have isolated two major proteins from released flagella of C. crescens CB13. The proteins (A and B) have molecular weights of 26,000 and 28,500 respectively, and have similar amino acid compositions. Whether the flagella consist of two subunit proteins or whether flagellar filaments of different subunit proteins are formed simultaneously in the cell cycle is not known.

Molecular weight of flagellin

Weibull (1948, 1950) employing Archibald's method with acid-dissociated preparations, first estimated the molecular weight for flagellin in P. vulgaris, B. subtilis and S. paratyphi B to be approximately 40,000. Later studies by Kobayashi, Rinker and Koffler (1959) on acid-dissociated preparations of flagellins of P. vulgaris Serratia marcescens, B. subtilis and three additional Bacillus species gave molecular weights of 14,000 to 20,000 among the different species. These values were obtained by use of ultracentrifugation and N-terminal amino acid analysis. Erlander, Koffler and Foster (1960) ascribed the higher value for P. vulgaris obtained in earlier investigations to the dimer formation of flagellin molecules under the experimental conditions employed. However, Chang, Brown and Glazer (1969) maintained that as

most of the determinations yielding a molecular weight value of 20,000 were performed by Erlander, Koffler and Foster in solutions of low ionic strength at a pH several units away from the isoelectric point, it is probable that the low value 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 charge effects. Chang, Brown and Glazer (1969) obtained a value of approximately 40,000 for P. vulgaris flagellin using sedimentation equilibrium in 6M guanidine in the presence of mercaptoethanol at pH 8.5. A similar value, 41,000, was also obtained for P. vulgaris flagellin by Parish and Marchalonis (1970) using polyacrylamide gel electrophoresis (pH 4.0, 9M urea) at concentrations ranging from 7-14% (w/v). Glossman and Bode (1972) reported a molecular weight value of 41,500 $\pm$ 2,500 for P. mirabilis using sodium dodecyl sulphate (S.D.S.) polyacrylamide gel electrophoresis. A molecular weight of about 25,000 for B. subtilis flagellin was obtained when the sedimentation constant measured at pH 2 or 3 was used for the calculation of molecular weight; a value of 40,000 was obtained when an alkali-dissociated preparation was dialysed against triethyl ammonium-NaCl and the molecular weight was calculated from sedimentation equilibrium data (Martinez, Brown and Glazer, 1967). The validity of the higher value was supported by amino acid analysis and fingerprinting analysis of the flagellin. The lower value was attributed to the low sedimentation constant of flagellin denatured under the experimental conditions employed for the centrifugation (Martinez et al., 1968). Mirsky (1970) has reported a molecular weight for B. megaterium KM of 33,400 to 34,400 using sedimentation equilibrium measurements, or 33,000 $\pm$ 500 using S.D.S. polyacrylamide gel electrophoresis.

In S. typhimurium, molecular weight determinations calculated from ultracentrifugation data on flagellin preparations obtained by various

dissociation procedures gave a consistent value of approximately 40,000. McDonough (1965) obtained a value of about 40,000 for urea-treated flagellin, calculated either by Archibald's approach to equilibrium method, or from sedimentation and diffusion data. Asakura, Eguchi and Iino (1964) obtained a similar value using acetone-dried flagella dissolved in 3mM phosphate buffer solution, pH 7.2. Gel filtration on a Sephadex G-100 column, eluted with phosphate buffer was used by Shinoda et al. (1970) to determine the molecular weights of the two component subunits (U-I and U-II) of V. parahaemolyticus flagella. (The subunits were firstly separated by elution from a hydroxylapatite column with phosphate buffer). A molecular weight of 40,000 was determined for each subunit. More recently, S.D.S. polyacrylamide gel electrophoresis has been the method of choice for molecular weight determination. However, values obtained do not always agree with those obtained by other methods. Tauschel (1970) reported a molecular weight value for Rhodopseudomonas palustris flagellin of 15,500 using the analytical centrifuge and 93,000 by S.D.S. polyacrylamide gel electrophoresis. Joys and Rankis (1972) obtained a molecular weight value of 49,000 for S. typhimurium, both by S.D.S. polyacrylamide gel electrophoresis and by gel filtration on agarose in 6M guanidine HCl-2-mercaptoethanol, a value not in agreement with previous estimations.

Comparison of molecular weight values of E. coli flagellin with Salmonella flagellins, as determined by S.D.S. polyacrylamide gel electrophoresis, revealed relatively large differences (Kondoh and Notani, 1974). E. coli flagellin had an apparent molecular weight of about 60,000, whereas Salmonella flagellin values varied from 51,000 to 57,000 depending upon antigenic type. This variation in molecular weight was found to occur not only between genera, but also between different species of a genus and different strains of a species (McDonough and Smith, 1976). Molecular weights ranged from 28,600

for B. sphaericus to 63,200 for one strain of E. coli using this method.

Amino acid composition of flagellin

The amino acid composition of flagellin was analysed in P. vulgaris (Koffler, Kobayashi and Mallet, 1956; Kobayashi, Rinker and Koffler (1959), several different serotypes of Salmonella (Ambler and Rees, 1959; McDonough, 1965), B. subtilis and S. serpens (Martinez, Brown and Glazer, 1967). Although the composition of flagellins from different organisms differs, they have several features in common. One such feature is the absence of cysteine, which is particularly interesting as flagellins have been likened by X-ray diffraction analysis to the actomyosin complex of skeletal muscle. The muscle protein, however, contains cysteine. The presence of cysteine in the flagellin of P. vulgaris and B. subtilis (Koffler, Kobayashi and Mallet, 1956; Kobayashi, Rinker and Koffler, 1959) was later disclaimed by these investigators (cited in McDonough, 1965). These later results were confirmed by Chang, Brown and Glazer (1969). Tryptophan is also absent from flagellin, whereas aspartic acid, alanine, glutamic acid and threonine are remarkably abundant; their sum exceeds half of the total amino acid residues of the molecule. Glycine, leucine, valine, lysine, serine, isoleucine, arginine, tyrosine, phenylalanine and methionine are also present. Proline and histidine are present in small amounts or are absent.

An unusual amino acid, ϵ-N-methyllysine (NML) has been reported in Salmonella flagellins of some, but not all, serotypes (Ambler and Rees, 1959). Using phase-mediated transduction studies, Stocker McDonough and Ambler (1961) have located a gene which determines the presence or absence of NML in flagellar protein. This gene is located very close to, but separable from the H-1 structural gene (Konno et al., 1976). However, Tronick and Martinez (1971), examining the methylation of endogenous proteins by S. typhimurium, revealed the

presence of at least two genes specifying different methylating enzymes. One gene product is a flagellin-specific methylating enzyme, whereas the other gene(s) codes for enzymes that methylate one or more other cell proteins. Quantitative replacement of NML by lysine is found in bacteria, the flagella of which do not possess NML (McDonough, 1965).

In growing cultures of S. typhimurium, flagellar NML can be isotopically labelled with either L - (Methyl - ^{14}C) methionine or L - (U - ^{14}C) lysine (Kerridge, 1963, 1966). Isotopic labelling of flagellar NML was unaffected by the addition of DL - NML to the incubation medium, but was competitively inhibited by the methionine analogues D-methionine and DL-ethionine; the growth rate of S. typhimurium was unaffected. Kerridge concluded that methylation of lysine residues probably occurred after their incorporation into the flagellin molecule, but was unable to obtain a cell-free preparation capable of synthesising NML. Comb, Sarker and Pinzino (1966) found that the addition of an energy source, amino acids or tRNA were not required for methylation of lysine residues in S. typhimurium, thus supporting Kerridge's theory.

NML residues have also been found in nuclear histone (Allfrey, Faulkner and Mirsky, 1964; Murray, 1964), S. serpens (Martinez, 1963b; Glazer, Delange and Martinez, 1969) and several P. morganii strains (Barr, 1973). However the NML : lysine ratio is only about 1:8 in S. serpens and 1:3 in P. morganii, compared to about 1:1 in Salmonella. In addition to mono-NML, Paik and Kim (1967) identified both di- and tri-NML in histones. Tri-NML residues have also been detected in several cytochromes (DeLange, Glazer and Smith, 1969). Using similar fractionation techniques, Glazer, DeLange and Martinez (1969) found about one half of a residue of di-NML per mole of Salmonella flagella. This finding suggests that the control of the degree of methylation may not be absolutely stringent.

In order to define which lysine residues were methylated in Salmonella flagellin, Joys and Kim (1978) examined soluble tryptic peptides, isolated from the phase-1 flagellin of S. typhimurium, for their NML content. The results demonstrated that only 5 of the 20 peptides isolated contained NML and that, in these cases, methylation was 98+% efficient. They concluded, therefore, that methylation of flagellin occurred with high efficiency at specific lysine residues.

McDonough (1965) carried out a detailed analysis of the amino acid composition of antigenically distinct Salmonella flagellins. All the flagellins contained a high proportion of aspartic and glutamic acids (or amides), threonine and alanine. Little tyrosine, phenylalanine, proline and methionine, and no cysteine, cystine or tryptophan were detected. The g..... series of Salmonella antigens contained no histidine; in the other flagellins it is the least abundant amino acid. The amino acid composition of the flagellins examined is similar to that of P. vulgaris (Kobayashi et al., 1959; Chang, Brown and Glazer, 1969) and S. typhimurium (Ambler and Rees, 1959; Joys and Rankis, 1972). In two respects, Proteus flagellin is more similar to the g..... than to the other Salmonella flagellins: that is, absence of histidine, and a phenylalanine to tyrosine ratio (about 1.4:1) approximately the reverse of those of the other Salmonella flagellins (0.6:1). Small amounts of proline were found in all the Salmonella flagellins examined, whereas none is reported in Proteus flagellin. Cross-reacting antigens were similar in amino acid composition, whereas comparison of pairs of serologically unrelated flagellins indicated a minimum amino acid substitution of 11-38 in the approximately 380 residues of the polypeptide chain. From these results, it was concluded that the amino acid composition of a particular antigenic type of flagellin is constant. The finding that amino acid differences existed between phase-1 flagellins of two S.

typhimurium strains of the same serotype (Joys et al., 1974) proved to be an exception to McDonough's conclusion.

Analyses of other flagellins reported include B. subtilis (Martinez, Brown and Glazer, 1967; Chang et al., 1976) and S. serpens (Martinez, Brown and Glazer, 1967) which both contain histidine and proline, and V. parahaemolyticus (Shinoda et al., 1970), whose two subunits (U-I and U-II) have very different amino acid compositions. Significant differences in lysine, aspartic acid, glutamic acid, alanine, valine and methionine contents were observed. The presence of histidine, and absence of proline and cysteine were noted in both V. parahaemolyticus and V. alginolyticus flagellins. Comparison of the amino acid compositions of the U-II of V. parahaemolyticus and the flagellin of V. alginolyticus (Miwatani and Shinoda, 1971) revealed slightly more methionine and serine in the U-II subunit and slightly more arginine in the flagellin of V. alginolyticus. A compilation of amino acid analyses of various proteins including flagellins has been published by Kirschenbaum (1975).

Cleavage of Flagellin

Enzymic cleavage

Peptides produced by enzymic cleavage of a protein molecule can be separated by two-dimensional peptide mapping, employing chromatography and electrophoresis, to give a distinctive pattern characteristic of the protein. This is also referred to as "finger printing". Trypsin is commonly used because of its specificity in cleaving only on the carboxyl side of lysine or arginine residues. Tryptic digestion was used by Hunt and Ingram (Ingram, 1959) to compare haemoglobin from normal adults and from patients suffering from sickle cell anaemia. The "fingerprints" revealed a difference of only one peptide between the two proteins. This was found to be due to the replacement of a glutamic acid residue of normal haemoglobin by valine in sickle cell

haemoglobin. Similar analyses of tryptic digests of flagellins showed the presence of 30 to 35 peptides in several different antigenic types of Salmonella flagellin (McDonough, 1962; Iino, 1964; Enomoto and Iino, 1966; Yamaguchi and Iino, 1969), and in B. subtilis flagellin (Martinez, Brown and Glazer, 1967). In S. serpens flagellin there were 36 to 37 peptides (Martinez, Brown and Glazer, 1967); 31 peptides were distinguished in P. vulgaris flagellin (Chang, Brown and Glazer, 1969). These numbers are consistent with the number of lysine and arginine residues per flagellin molecule estimated by taking the molecular weight of these flagellins to be about 40,000.

In at least 4 of the 7 serologically mutant forms of the i flagellar antigen of Salmonella analysed by McDonough (1962), 1 peptide out of the total of 30 was found to differ from the wild-type. Iino (1964) showed that flagellins of enx- and 1,2- antigens differed in 6 among 35 component peptides, whilst a curly mutant and its wild-type differed in at least one peptide. A single peptide difference was also reported between flagellin of normal and straight flagella of B. subtilis (Martinez et al., 1968). This was identified as the replacement of alanine by valine in the altered peptide of the straight mutant.

Cyanogen bromide cleavage

Cleavage of flagellin molecules by chemical means has also been employed. Parish and Ada (1969) used cyanogen bromide to cleave S. adelaide flagellin at methionine residues. Since the flagellin has three methionine residues, this resulted in the production of four fragments (A, B, C and D). Three of the four fragments, A, B and D, were isolated in pure form and their amino acid compositions determined. The C-terminal sequence of the flagellin was established as Leu-Leu-Leu-Arg, and a tentative alignment of the fragments in the intact molecule was suggested. This proposed sequence was confirmed by Davidson (1971)

by determination of the amino acid sequences of methionine-containing tryptic peptides, and from the results of end group analyses on the cyanogen bromide fragments. Similarly, Glossman and Bode (1972) cleaved P. mirabilis flagellin with cyanogen bromide, producing three relatively insoluble peptide fragments that could be dissolved by subsequent maleylation.

Alignment of the cyanogen bromide peptides of P. mirabilis and S. adelaide flagellins:-

	N-terminal								C-terminal
	Fragment	P3			P2			P4	
P. mirabilis	Ala	———	Met	Phe	———	Met	Val	———	Leu Arg
	M. Wt.	12,500			23,500			5,000	

	Fragment	B		A		D		C	
S. adelaide	Ala	—	Met	Lys	—	Met	Phe	—	Met Ser — Leu Leu Leu Arg
	M.Wt.	12,500		18,000		4,500		5,500	

Using a combination of enzymic and chemical digestion, certain flagellins have been fragmented and partial amino acid sequences determined. B. subtilis 168 flagellin has been completely sequenced (DeLange et al., 1973; Chang et al., 1976), but only limited information is available on the primary structure of flagellin of S. typhimurium (Joys and Rankis, 1972), S. adelaide (Davidson, 1971) and P. mirabilis (Glossman and Bode, 1972). However, comparison of the complete and partial amino acid sequences is highly suggestive (DeLange et al., 1973). For example, the carboxyl terminal residues of B. subtilis (DeLange et al., 1973; Chang et al., 1976), S. adelaide (Davidson, 1971) and P. mirabilis (Glossman and Bode, 1972) flagellins are Leu-Arg and close homology may well extend considerably beyond these residues. The fragmentary information on the tryptic peptides of S. adelaide (Davidson, 1971) and S. typhimurium (Joys and Rankis, 1972) is strongly suggestive of homologies between the sequences of

of these proteins and the flagellin of B. subtilis (DeLange et al., 1973).

(ii) The Unit of Flagellar Antigen

The presence of flagella-specific antigens was first recognised in S. choleraesuis by Smith and Reagh (1903). Weil and Felix (1917) gave the symbols O and H to nonflagellated and flagellated types of bacteria, respectively, based on the form of their colonies, namely, spreading (Hauch) and nonspreading (Ohne Hauch). The use of these symbols has now been extended; O refers to the somatic antigens and H to the flagellar antigens. Early evidence for this comes from Craigie (1931) who showed that the floccular agglutination of B. typhosus by H antisera is dependent on the presence of flagella. When the flagella are removed by violent shaking or by heating, there is no longer any agglutination with H antisera. Also purified flagella are agglutinated by H antisera but not by O antisera.

The specificity of the flagellar antigenic type has been extensively surveyed in Salmonella on a worldwide scale, and an abundance of well-determined antigen types classified in the Kauffman-White Scheme (Kauffman, 1954, 1965). This scheme is limited to studies on bacteria belonging to the Enterobacteriaceae. However antigenic specificity has also been observed in flagella of different species of Spirillum and different strains of B. subtilis (Martinez, Brown and Glazer, 1967). The subunits U-I and U-II isolated from the flagella of V. parahaemolyticus were shown to be antigenically different (Shinoda et al., 1970). Miwatani and Shinoda (1971) demonstrated antigenic similarity between flagellin of V. alginolyticus and the U-II subunit of V. parahaemolyticus.

The antigenic type of a particular kind of flagellin is a complex of antigen type determinants, although for descriptive convenience they are often indicated by a simplified symbol (Kauffman, 1954). This

determinant complex is carried as a unit not only by a single flagellum, but also by a single molecule of flagellin. The former was first shown by Nakaya, Uchida and Fukumi (1952) who demonstrated that the antiserum for antigen subunit g or m of S. enteritidis agglutinated all isolated flagella of this bacterium. Ada et al. (1963) obtained rabbit antisera for flagellin, flagellar fibres and flagella-like filaments produced by reaggregation of the flagellin of S. adelaide. Each antigen was effective in neutralising antibody prepared against any one of the antigens, and gel diffusion and immunoelectrophoresis showed one main common antigen with sometimes a second minor antigen. In B. subtilis and S. serpens, only one precipitin band has ever been observed when homologous flagellins are used as antigens in gel diffusion tests (Martinez, Brown and Glazer, 1967). However, it should be pointed out that a different purification method was used in each case.

Thus the flagellin molecule appears to constitute a unit of antigen carrying the common specificity of the flagella. However, results from complement fixation tests carried out by M.I. Simon (cited in Joys, 1968) suggest that the situation might be more complex. 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 anti-flagellin/flagellin reaction. Joys concludes from these results that a flagellum and its component flagellin contain common sites of antigenic specificity and sites of difference. The flagellin-specific 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. Ichiki and Martinez (1969) using immobilisation-inhibition and DEAE cellulose binding assay to compare the antigenic properties of B. subtilis flagella and periodate-treated flagellin, suggested that there were four types of antibodies elicited by flagella and flagellin, of which two were unique and two were cross-reacting.

The presence of common and variable antigenic determinants is not restricted to a flagellum and its component flagellin. Langman (1972) has demonstrated the occurrence of antigenic determinants common to flagella of different Salmonella strains which had hitherto been regarded as antigenically distinct. Antibodies to the common antigenic determinants (Hc) could be demonstrated by the Ouchterlony gel diffusion technique and by passive haemagglutination assays, but not by bacterial immobilisation assays. To account for these results Langman proposed that the variable antigenic determinants (Hv) are distributed along the flagella so that antibody can bind with two sites on the one flagella, thus disrupting the wave motion; in contrast, antibody to Hc determinant can only bind to one flagellum at a time and therefore acts as a cross-link, and does not cause immobilisation.

Comparative amino acid analysis of flagellins of antigenically distinct Salmonella flagella showed that the antigenic difference is associated with the difference in amino acid composition between flagellin molecules (McDonough, 1965; Yamaguchi and Iino, 1969). This finding led to the conclusion that the specificity of a flagellar antigen is a reflection of the surface conformation of a flagellin molecule, which is primarily characterised by specific amino acid sequence. Precipitin-inhibition tests with the fragments obtained after cleavage of flagellin with cyanogen bromide (CNBr) further indicated

that the polypeptide region responsible for antigenic specificity is localised on a flagellin molecule (Ada et al., 1967; Parish and Ada, 1969). Further work by Parish, Wistar and Ada (1969) established that the largest CNBr polypeptide, fragment A, molecular weight 18,000, contained all the antigenic specificities present on flagellin. Ichiki and Parish (1972) partially cleaved S. adelaide flagellin with pepsin at sub-optimal pH (pH 5.0) or with trypsin at sub-optimal temperature (25°C). Peptic digestion produced 3-4 large fragments with molecular weights between 13,000 and 15,000. Tryptic digestion released 2 large trypsin resistant peptides with molecular weights between 16,000 and 18,000. Serological tests demonstrated that most of the antigenic determinants of flagellin were localised in the pepsin and trypsin resistant fragments, although there was some indication of loss or weakening of one or more determinants. Ichiki and Parish concluded that the pepsin resistant fragments were derived from within the fragment A portion of flagellin, whereas the trypsin resistant peptides represent an overlap between fragment A and some other CNBr fragment.

Mutants of S. typhimurium with serologically altered i antigens have been isolated (Joys, 1961; Joys and Stocker, 1963; 1966) and shown to possess chemically altered flagellin molecules, as indicated by differences in peptide maps (McDonough, 1962). These mutants possessed new, unique specificities and retained some of the original wild-type specificities. Yamaguchi and Iino (1969; 1970) attempted to map the antigenic specificity-determining sections in the H1 structural gene of S. abortusequi by intragenic recombination between different alleles of of the H1-g.... complex. The genetic, serological and peptide pattern of these recombinants resulted from a recombination of parental properties.

The marked variation observed in the amino acid sequence of Salmonella flagellins has also been shown to extend to B. subtilis

(Emerson and Simon, 1971). The flagella from 18 strains of B. subtilis were tested for their reaction with anti-flagellar filament antibody and anti-flagellin antibody. On the basis of their reactivity, at least 5 serologically distinct classes could be identified. Peptide map analysis of tryptic digests of the subunit proteins were consistent with the immunochemical analysis. Large differences in sequence existed among proteins of the different classes, whereas proteins within an antigenic group differed by only a few peptides.

Methylation of lysine in flagellin has been shown to be serologically important in certain cases (Stocker et al., 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 introduction of the nml^+ gene, antigen 3 appears as well as 1,2, and vice-versa (Joys and Stocker, cited in Pearce, 1965). However, no changes in the e,n,x antigens of S. abony were detected with change in the presence of NML.

(iii) Reaggregation of flagellin molecules in vitro

Spontaneous polymerisation

Erlander, Koffler and Foster (1960) reported that when P. vulgaris flagellin was exposed to a certain concentration of salt, it precipitated and formed a stable aggregate polymer. This was one of the first reports of reaggregation of flagellin molecules in vitro and, subsequently, this process was found to occur under a variety of conditions. The flagella-like filaments formed were considered to be indistinguishable from native flagella.

Polymerisation of Salmonella flagellin following ammonium sulphate precipitation has been reported by Ada, Nossal, Pye and Abbott (1963); Lowy and McDonough (1964); and Wakabayashi, Hotani and Asakura (1969). Asakura, Eguchi and Iino (1964; 1966; 1968) have assumed that the polymerisation process is similar to crystallisation and thus flagellin

solutions will only polymerise on the addition of seeds. Abram and Koffler (1964) have shown that B. pumilis flagellin polymerises into flagellar filaments at low ionic strengths without the addition of either seeds or high concentrations of salt. Electron microscopy of flagellin aggregates from B. pumilis revealed three forms. At high temperature (38°C) and below pH 5.4, amorphous material is formed which is presumed to be a precipitate of denatured protein. At temperatures below 26°C, straight reaggregates are formed within a pH range of about 4.0 to 4.9, and flagellum-like filaments are formed between pH 5.3 and 6.2. The latter are morphologically indistinguishable from native flagella. Straight filaments can be transformed to flagellum-like filaments if they are resuspended at pH 5.4; this transformation is irreversible. The spontaneous reaggregation of flagellin to flagellum-like filaments was also observed on B. stearothermophilus (Abram and Koffler, 1964) and on B. subtilis (Martinez et al., 1968).

In contrast to Bacillus flagellins, monomers of Salmonella flagellin can reaggregate and form flagellum-like filaments at high concentrations of an appropriate salt, for example $(NH_4)_2SO_4$, at neutral pH (Ada et al., 1963; Lowy and McDonough, 1964). Electron microscopy revealed the very close similarity between the structure of the reassembled filaments and that of the native flagella attached to the bacterium (Lowy and McDonough, 1964). A more detailed study by Wakabayashi, Hotani and Asakura, 1969), using heat-depolymerised flagellin, revealed that polymerisation can be initiated by the addition of moderately high concentrations of anions F^-, CO_3^{2-}, HPO_4^{2-} and citrate ions which are known as good "salting-outers". By contrast, in the presence of high concentrations (more than 0.5M) of Mg^{2+} and Ca^{2+}, which are good "salting-inners", flagella undergo depolymerisation. The average length of reformed flagella, measured after complete polymerisation, decreased with increasing concentrations of the above

anions and with increasing concentrations of flagellin. In addition, some of the short filaments took on a straight form. X-ray diffraction of reconstituted Salmonella flagella which have the straight form, revealed no major difference between their structure and that of normal flagella (Wakabayashi and Mitsui, 1970).

Requirement of seeds

In a neutral salt solution, monomeric flagellin prepared by heat treatment is apparently stable and remains in a state of super saturation (Asakura, Eguchi and Iino, 1964). To polymerise the monomers in such solutions, it was found necessary to add fragmented flagella prepared by sonication. Then the ends of added fragments act as nuclei, resulting in the rapid formation of long flagellar filaments; the process is characteristic of crystallisation (Asakura, Eguchi and Iino, 1964). When monomer and seed are mixed in protein ratio (r), the average length of filaments fully grown in the mixture is (r + 1) times the average length of added seeds. This means that during polymerisation the number of filaments contained in the solution remains unchanged and a one-to-one correspondence exists between the added seeds and the fully grown filaments.

Copolymerisation

For initiating polymerisation of a given kind of monomer, seed need not necessarily be homologous with it (Asakura, Eguchi and Iino, 1966). For instance, monomers and seeds derived from Salmonella strains of different antigenic type and flagellar shape can be mixed in all combinations for initiation of polymerisation. When the protein ratio between the mixed monomer and seed is large, the overall rate of polymerisation is determined by the nature of monomer. In early periods of polymerisation, the rate of polymerisation depends upon the nature of added seed. The addition of seed to a mixture of two kinds of monomer can lead to the copolymerisation of monomers (Asakura, Eguchi

and Iino, 1966). Copolymerisation of about equal concentrations of normal-type monomer and curly-type monomer resulted in the formation of curly-type filament, irrespective of the nature of added seed. Also, the normal type filament was sometimes transformed into curly when it was left standing for long periods. The transformed filaments could be reversed into normal by the addition of a few mM of ATP or pyrophosphate at neutral pH. These observations led to the conclusion that flagellar filaments are dimorphic in nature. Kuroda (1972) has shown that cross-interaction between monomers and seeds, and copolymerisation of two kinds of monomers occur not only within Salmonella but also between Salmonella and Proteus. On the other hand, between Salmonella and Bacillus or Proteus and Bacillus, cross-interaction and copolymerisation were unsuccessful.

Rate of Polymerisation

Gerber and Noguchi (1967) measured the rate of the volume change of the solution associated with the polymerisation of flagellin to flagella in Salmonella and found it to be temperature-dependent. The effect of increasing temperature was explained by the assumption that the protein is reversibly transformed to a state which lacks the capacity to reconstitute flagella; above 28°C, this transformation becomes rate-limiting for polymerisation. This hypothesis was supported by investigations made by Gerber, Asakura and Oosawa (1973) on the temperature dependence of the rate of polymerisation and depolymerisation. An unexpected finding was that the temperature defines a critical monomer concentration which exists in equilibrium with any concentration of filaments. This equilibrium feature of polymerisation is an essential element in common with crystallisation and is incorporated into a theory proposed by Oosawa and Higashi (1967) on the formation of tubular polymers of proteins.

At a low concentration of NaCl and neutral pH, both polymerisation

and depolymerisation rarely proceed for long periods. Therefore, rapid polymerisation initiated in the presence of 0.15M NaCl can be stopped at any time by diluting the solution, and flagellar filaments separated by centrifugation. Thus the increase (or decrease) in a unit time of the concentration of flagellar filaments (or monomer) during rapid polymerisation can be determined (Asakura, 1968).

The initial rate of polymerisation seems to saturate to a final level on increasing the concentration of monomer and obeys a relation of the Michaelis-Menten type. One of the possible interpretations of these results is to assume that polymerisation consists of two steps. The initial step corresponds to reversible binding of a monomer onto an end of an existing filament, and the second step corresponds to the incorporation of the bound monomer into the filaments; only after incorporation can the bound monomer start to act as a new end for further polymerisation. Whatever the mechanism of incorporation, the bound monomer is supposed to undergo some conformational change during this process. This was investigated by Uratani, Asakura and Imahori (1972) who carried out a circular dichroism (C.D.) study of Salmonella flagella. C.D. spectra from the monomer and polymer solutions were approximately similar in shape with a minimum at 222mμ, indicative of an α-helical structure. However, the C.D. of polymer is much larger in magnitude than that of monomer. This was not due to the fact that polymer solutions used in the experiment were inevitably more turbid than monomer solutions, but was attributed to a change in secondary structure on polymerisation of flagellin into flagella. Similar studies were made on P. mirabilis flagellin (Bode and Blume, 1973).

The physical basis for the high specificity involved in polymerisation was sought in a study of the dielectric behaviour of the monomer and polymer form of S. abortusequi flagellin (Gerber, Routledge and Takashima, 1972). The monomer had a permanent dipole moment

greater than 700 Debye units; on polymerisation this value decreased markedly. Since the dipole moment is a vector quantity, it was suggested that neutralisation of permanent dipole moments may dictate the morphology of the polymer and/or the rate of its assembly. A more complete study was made by Gerber, Minakata and Kahn (1975) using the technique of electric birefringence to measure the rate and extent of orientation of molecules in response to a pulsed electric field. The results were interpreted as a side-wise aggregation of bacterial flagellar filaments. Thus variations in electric potential along the membrane of the bacterium might serve first to orientate these organelles and then to induce their aggregation into bundles.

Unidirectional growth

By using flagellin monomers and seed segments antigenically distinct from each other, and staining the reconstituted filaments with antiserum specific for monomer or fragment, it was demonstrated that the reconstitution proceeds from one end of the fragmented flagella (Asakura, Eguchi and Iino, 1968).

Electron microscopy of fragments of Salmonella flagella presented by Poglazov (1966) indicates that the end with a fish-tail shape (T-end) corresponds to its distal end when attached to the cell. Abram, Koffler and Vatter (1966b) obtained similar results with isolated Bacillus flagella. Furthermore, the distal end was found to correspond to the end where flagellar growth takes place both in vitro (Asakura, Eguchi and Iino, 1968) and in vivo (Iino, 1969b). However, Pye (1967) reported that when washed Salmonella cells with short flagella were added to a solution of monomeric flagellin, the monomer did not polymerise on to the distal ends of these flagella. He concluded that the growth of flagella occurred at only one end and assumed this to be the proximal or H-end of the flagellum.

Oosawa and Higashi (1967) have presented a theory on the mechanism

of the directional polymerisation of proteins: this theory is based on the assumption that proteins undergo conformational change during polymerisation. They showed that this property of protein necessarily gives rise to directionality in polymerisation or growth. Models proposed for the structure of flagella have also suggested a structure with inherent polarity, in agreement with the above observations (Gerber, Routledge and Takashima, 1972; O'Brien and Bennett, 1972; Bode et al. 1972).

Melting of flagellar polymers by heating was also shown to be unidirectional (Hotani and Kagawa, 1974). By cross-mixing monomers and seeds derived from two different antigenic strains of Salmonella, n and i, two types of block copolymers were prepared; (n-i) and (i-n). After partial melting of the copolymers at 50°C, the products were labelled with antiserum against n-flagella and examined by electron microscopy. On the basis of the statistical analyses of the data with the aid of computer simulations, it was concluded that, upon heating, flagellar polymers melt almost exclusively at their T-ends.

Length of reconstituted filaments

A flow birefringent study by Fujime et al. (1972a) suggests that the mean length of bacterial flagella of a Salmonella strain reconstituted in vitro does not exceed some critical length, say, approximately 6μm. Since the monomers remaining in solution can polymerise upon addition of new seeds, the self-limiting mechanism of polymerisation seems to be intrinsic. Hotani and Asakura (1974) showed that Salmonella filaments stopped growing or became inactive for a long period of time when monomer and seed were mixed in a protein ratio (r) larger than 20. The average length of the inactive filaments, however, was independent of the value of r. They called this phenomenon, "growth-saturation".

If growth of flagella occurs at the distal end, then flagellin,

which is undoubtedly synthesised inside the cell, must be transported from the cell to the point of growth. Diffusion of newly synthesised flagellin is highly improbable. Flagellin might be transported through the central hole of the flagellum. Interestingly, Iino, Suzuki and Yamaguchi (1970; 1972) reported that even when flagella are attached to living cells, reconstitution of flagellar filaments from exogenous flagellin monomers occurs at their tips under appropriate conditions. In addition, the reconstituted flagella can exert their locomotive function.

Extraordinary polymerisation of flagellin molecules in vitro: P-Filaments

Hotani, Asakura and Iino (1969) found that polymerisation of Salmonella flagellins takes place when membraneous components extracted from deflagellated cells were added to the flagellin solution, producing long, straight filaments that differ in morphological and physiological properties from the original flagella. Electron microscopic observations revealed that these filaments are straight, but not uniform in thickness, and are rough in surface appearance. The average thickness is less than that of normal flagella (Hotani, 1971; Kagawa, 1973). They are remarkably stable when exposed to heat, and to both acid and alkali. They were named P-filaments by Hotani et al. (1969) because of their similarity in appearance to bacterial pili. Similar filaments were formed by adding bile salts instead of the membraneous components to the flagellin solution (Kagawa, 1973). Biochemical analyses (Hotani et al., 1970; Kagawa, 1970) revealed that neither the added membraneous components nor the bile salts were contained in the structure of the P-filaments, and that the flagellin in the P-filament had the same primary structure as that of the original flagella. But studies by means of optical rotatory dispersion (Hotani, 1971) and circular dichroism (Kagawa, 1973) suggested that they had different secondary structures. In addition, P-filaments

produced by the above procedure can be disaggregated, by adding a concentrated solution of guanidine hydrochloride, into flagellin molecules and repolymerised to give the original flagellar structures (Hotani, 1971; Kagawa, 1973). X-ray diffraction of P-filaments (Wakabayashi et al., 1974) revealed no regular layer-line reflection suggesting that practically no order exists in the arrangement of the flagellin molecules. Bacterial pili, on the other hand, have an ordered structure with respect to subunit arrangement (Mitsui, Dyer and Langridge, 1973). Thus, at this molecular level there is no structural similarity between P-filaments and bacterial pili. In addition, the cross β-structure is predominant in the P-filament, but the original flagella have virtually no β-structure.

2. Growth of flagella *in vivo*

a) Flagellation and Cell Cycle

The distribution of the number of flagella per cell is characteristic for each bacterial strain under a given cultural condition. During a cell cycle of peritrichously flagellate bacteria, the number of flagella doubles and upon cell division they distribute to daughter cells approximately evenly. This indicates that the formation of flagella is genetically regulated so as to be coupled with the cell cycle.

Synchronous cultures of *B. subtilis* 168 were obtained from light-density spores germinated at 46°C and grown at 37°C; this procedure synchronises both cell division and chromosome replication. In such bacteria, the doubling of the rate of flagellar formation was found to correspond to the time of replication of the his Al gene (Van Alstyne et al., 1969). The synchronisation of flagellar formation in a synchronised culture of *E. coli* was also reported (Kondoh and Ozeki, 1973). Several temperature-sensitive mutants of *E. coli* defective in DNA replication, for example, fts and dna, are retarded in flagellar

formation at a nonpermissive temperature (Nishimura et al., 1975). These observations strongly suggest that the initiation of flagellar formation is coupled with DNA replication, although little is known about the coupling mechanism. A regulatory factor, adenosine 3', 5'-cyclic phosphate (cAMP), and the protein specifically bound to it (CRP) are presumed to be involved in the initiation of flagellation. Either cAMP-deficient mutants (cya^-) or CRP-deficient mutants (crp^-) of both E. coli and S. typhimurium are defective not only in sugar fermentation, but also in flagellar formation (Yokota and Gots, 1970; Silverman and Simon, 1974b; Komeda et al., 1975). Thus the complex of cAMP and CRP has a regulatory function for both sugar fermentation and flagellation. Suppressor mutants (constitutive flagella synthesis (cfs)) have been isolated which restore flagella-forming ability, but not sugar fermentation in cya^- and crp^- mutants (Silverman and Simon, 1974b; Komeda et al., 1975). cfs is dominant over its wild allele. The mutant site of cfs was mapped in fla I in E. coli and fla T in S. typhimurium (Silverman and Simon, 1974b; Komeda et al., 1975). The step of flagellar formation sensitive to cAMP may be at a very early stage of flagellar formation because none of the precursor structures of flagellar bases were detected on cya^- cells.

The role of cAMP in flagellation is explained as follows: cAMP receptor protein together with cAMP modulates the wild allele of the gene in which the cfs mutation resides, and the gene in turn acts as a positive effector on the initial step of flagellar formation. Then, the cfs mutant gene may be regarded as the constitutive effector.

b) Sequence of flagellar formation

A detailed electron microscopical study of cell envelope fractions of various nonflagellate mutants detected a series of precursor structures of flagella (Suzuki et al., 1976). The simplest precursor structure detected in nonflagellate mutants of Salmonella is the

complex of two inner rings and a rod (RIV particle). The next simplest is the complex of a RIV particle and a P ring (CAS particle). Then comes the complex of a CAS particle, a cylinder, and an L ring (BAB particle. A BAB particle is morphologically indistinguishable from a basal body of an intact flagellum. The precursor between BAB and the intact flagellum is the complex of a BAB particle and a hook (HOB particle). These precursor structures were cistron-specific. A diagram showing the sequential process of flagellar morphogenesis in Salmonella is given in a review by Iino (1977).

The existence of the complex of a rod and two inner rings as the first detectable structure suggests that flagellar formation starts from the assembly of inner rings associated with the cell surface layers. The dependence of flagellar formation on prior synthesis of a normal cell wall was first suggested by studies on spheroplasts (Vaituzis and Doetsch, 1966). Flagella of penicillin-induced spheroplasts of S. typhimurium were confined to those areas of the spheroplast where cell wall fragments remained. Spheroplasts produced from nonflagellate cells were incapable of forming flagella; upon inactivation of penicillin, flagella again were synthesised during reversion of the cells to their original rod form. A mutation that causes a defect in the cell surface layers will retard flagellar formation. Deep-rough mutants in S. typhimurium have no flagella (Ames et al., 1974). Flagellar formation in uridine diphosphoglucose pyrophosphorylase-deficient gal U^- mutants of E. coli is markedly reduced (Komeda et al., 1977). The lipopolysaccharide layer is incomplete and some kinds of membrane-bound proteins are released in these mutants (Ames et al., 1974; Fukasawa et al., 1963). In a mutant of B. subtilis, production of α-amylase, proteases and autolytic enzyme(s), and formation of flagella are pleiotropically affected, possibly by certain undetected alterations of the cell envelope

(Ayusawa et al., 1975). Preliminary observations indicated that mutants of cya, crp, fla I, fla H or gal U have no flagellar basal body (Suzuki, Komeda and Iino, unpublished data), thus it is probable that some components or structures of the surface layers are essential for the early stages of flagellar morphogenesis.

As flagellar formation proceeds outwards from the cell surface, transport of components synthesised in the cytoplasm to the outer surface layer is necessary. Identification of the transport systems involved has not yet been achieved.

A block in the main pathway of flagellar morphogenesis by a fla$^-$ mutation can result in the formation of polyhooks or superhooks. A polyhook is structurally homologous with a normal hook, but exceedingly longer (Kagawa et al., 1976). Polyhooks have been detected in fla E mutants of E. coli (Silverman and Simon, 1972) and fla R mutants of S. typhimurium (Patterson-Delafield and Martinez, 1973). The mutant allele is recessive to its wild-type allele. In these mutants, few polyhooks bear flagellar filaments. The product of the wild-type allele may control the length of the hooks and the initiation of filament growth, presumably acting as "terminator" for hook protein assembly and "initiator" for flagellin assembly at the tip of each hook.

c) Flagellin Pool?

The presence of a functional flagellin pool in bacterial cells was inferred from Ouchterlony or quantitative precipitation tests on cell lysates of P. vulgaris and B. subtilis (Weinstein et al., 1960; Nasser and Koffler, 1963), and also from regeneration experiments on flagella of B. subtilis and S. serpens (Martinez and Gordee, 1966). However, Kerridge (1963) studied the kinetics of isotope incorporation into flagella and concluded that a functional pool of flagellin was not present in S. typhimurium. This view was supported by Quadling and Stocker (1962) who observed the inhibition of flagellar regeneration

by chloramphenicol in S. typhimurium. Roberts and Doetsch (1966) however, observed, in several monotrichous bacteria, the regeneration of flagella in the presence of chloramphenicol at a concentration 100 times that required to inhibit cell multiplication. A flagellin pool was also detected by immunological double diffusion techniques in a mutant of B. subtilis in which production of exoenzymes and formation of flagella are pleiotropically affected.

d) Regulation of flagellin synthesis

The synthesis of flagellin, whose assembly occurs at the final step of flagellar morphogenesis, appears to be under the control of a complex regulatory system. So far, no flagellin cross-reacting material has been detected in any fla^- mutants of Salmonella (Iino and Enomoto, 1966). An exception to this rule is a mutant assigned as fla G in Salmonella. However, it was later found to carry paralysed flagellar filaments in less than 1% of the cells and its mutant site was mapped in fla AII (Suzuki and Iino, 1975).

Suzuki and Iino (1975) examined the messenger activity of extracted RNA from Salmonella for flagellin synthesis using an E. coli cell-free system for protein synthesis. When RNA extracted from the flagellate strains was used, flagellin synthesis together with the synthesis of other proteins took place. When RNA of the nonflagellate mutants belonging to at least 11 different complementation groups was used, no detectable flagellin synthesis occurred although other proteins were synthesised. This was also the case when mRNA from a cya mutant which had a defect in cAMP synthesis and consequently failed to produce flagella, was used (Komeda et al., 1975). Extracts of fla^- mutant cells do not inhibit the synthesis of flagellin directed by mRNA of fla^+ strains; furthermore, RNA-free extracts of fla^+ strains do not promote the synthesis of flagellin by mRNA of fla^- strains (Suzuki and Iino, 1975).

Thus not only flagellin but also mRNA specific for flagellin are not synthesised in any fla$^-$ mutant. Silverman and Simon (1973b) demonstrated, using bacteriophage mu-induced flagellar mutants of E. coli, that the structural gene for flagellin (hag) constitutes an operon independent of any other fla cistrons, the latter being distributed among several discrete operons. Therefore, there must be regulation between operons for the synthesis of flagellin mRNA to take place. It is possible that fla L of Salmonella acts as a "linker" between the induction system and the operons for flagellin synthesis (Iino et al., 1975). The fla L mutants can produce flagellar structures other than filaments, but synthesis of mRNA specific for both H1 and H2 is blocked simultaneously in the mutants. Furthermore, some revertants of fla L partially recover the ability to synthesise flagellin; the resulting partial revertant cells produce flagellar filaments shorter than those of the normal fla$^+$ cells. It is suggested that the product of fla L acts as an antirepressor of flagellin synthesis.

e) Growth of flagellar filaments

The process of assembly of flagellin to flagellar filaments has been clarified in detail by the in vitro polymerisation experiments with Salmonella flagellin (Asakura, 1970). A flagellin monomer binds to an end of an existing filament; the end corresponds to the distal end of a flagellar filament on living bacteria. Then the monomer is incorporated into the filament accompanied by its conformational change. The incorporated monomer can then act as a part of the nucleus for polymerisation of the next monomer to begin. Thus a conformational change of flagellin molecules upon assembly confers structural polarity to the flagellar filaments and limits attachment to the distal end of each filament. This "self-assembly" process proceeds in the presence of appropriate concentrations of flagellin monomers and seeds, at the

proper temperature, pH and ionic strength (Asakura, 1970).

The growth of flagellar filaments *in vivo* appears to be essentially the same as that *in vitro*: growth of a flagellar filament *in vivo* proceeds at the distal end (Iino, 1969b; Emerson et al., 1970; Iino et al., 1972); under optimal conditions the maximum initial rate of polymerisation *in vivo* is the same as that *in vitro*.

Although polymerisation of flagellin monomers *in vitro* will proceed without the presence of filaments under conditions of high ionic strength (Wakabayashi et al., 1969), the filament must be present for polymerisation to occur in the ordinary physiological environment. This implies that initiation of filament growth *in vivo* under ordinary physiological conditions involves a different process to that *in vitro*. Assembly of flagellin monomers *in vivo* begins at the distal end of each hook. In fact, isolated hooks were successfully used as a heteronucleus for the polymerisation of flagellin *in vitro*, although the efficiency of the process was low compared with the polymerisation in which fragmented flagellar filaments were used as the homonucleus (Kagawa et al., 1973; Iino, 1974). Moreover, the possibility was not excluded that these hooks might contain a small amount of flagellin at their ends.

A flagellar filament elongates *in vitro* at a constant rate as long as a sufficient amount of flagellin is supplied, and the elongation terminates by an error occuring at the growing end of the filament (Hotani and Asakura, 1974). On the contrary, the rate of *in vivo* elongation decreases exponentially with increase in length of filament (Iino, 1969b; 1974). This is true even among the filaments of various lengths growing on a single cell, or for a filament mechanically shortened by breakage. Therefore, the suggestion that ageing of a cell or of a flagellum-forming apparatus causes the decrease in the growth rate of filaments is not tenable. For the growth of a flagellar

filament, flagellin monomers must be transported from the cell body to the tip of the filament probably via its central canal (Iino, 1969b, Emerson et al., 1970; Iino et al., 1972; Iino, 1974). Consequently, it seems likely that the decrease in growth rate is caused by the decrease in the efficiency of transportation with the increase in length of the filament. This means that the filament limits its own growth rate, which could explain the observed maximal length of flagellar filaments on living bacteria. However, the possibility of a termination factor for filament elongation is not entirely excluded.

An interesting phenomenon as regards the length of bacterial flagella was reported by Weinberg and Brooks (1963), who examined the effect of various metal ions on flagellation. In cultures of B. subtilis grown in basal medium enriched with 6×10^{-4}M each of manganese and aluminium, less than 10% of flagellated cells were present, but the length of the flagella was increased as much as 10-fold over normal flagella. The mechanism of this effect is not yet explained.

3. Genetics of Flagella

a) Structural genes for flagellin

The flagella genes whose functions were most clearly identified are the H1 and H2 in Salmonella (Iino, 1969a), H in B. subtilis (Joys and Frankel, 1967) and Ps. aeruginosa (Iino, 1969c) and hag in E. coli (Armstrong and Adler, 1969). Not only antigen specificity, but also flagellar shape and sensitivity to flagellotropic phage are controlled by the flagellin genes.

That the antigenic specificity of flagella is determined wholly by the structural gene of flagellin was demonstrated by transduction analyses between different serotypes of Salmonella (Lederberg and Edwards, 1953). Genetic and biochemical studies of antigenic mutants have further confirmed this conclusion (Joys and Stocker, 1963; 1966).

The distribution of the sites of antigenic determinants in the H genes was investigated by both mutation and recombination studies. Systematic genetic mapping was first undertaken on phase-1 antigen i of S. typhimurium; five mutant sites were mapped linearly (Joys and Stocker, 1963). Further extensive absorption-agglutination tests on mutants with altered i antigens showed that at least 13 antigenic factors exist in the wild-type i antigen, and that each of the mutant antigens obtained lacked a different combination of these factors (Joys and Stocker, 1966). The possibility that the determinant sites of antigenic factors can be assigned linearly each as a unit in the H gene was suggested by Iino (1959), who, considering the factor composition of naturally occuring g-complex antigens, proposed a preliminary model dividing H1 into five sections, each of which is marked by an antigenic specificity. An experimental approach to the mapping of the component factors of g-complex antigens was made by Yamaguchi and Iino (1969), who demonstrated intragenic recombination between H1 alleles specifying various forms of the g-complex antigens, and mapped some antigenically important areas of the H1 gene in a linear array.

Later, a map of H1 was constructed by Horiguchi et al. (1975) with non-flagellate H1 mutants isolated from a phase-1 stable derivative of S. abortusequi. First, mapping was carried out with the deletion mutants among them by P22 phage-mediated transduction. As a result, H1 was divided into 16 segments by 15 deletions. Mapping by recombination frequencies was then carried out using representative H1 mutants. Comparison of the two maps showed that 14 consecutive segments near fla L covered about 70% of the non-flagellate H1 mutational sites, although they were confined to a quarter of H1 in the recombination map. Further, this region was shown to cover none of the antigenic specificity-determining areas. The other two segments were found to occupy the remaining three quarters of H1. An attempt was made to

allocate antigenic specificity-determining areas within H1; they were located between fla AI and a curly mutational site, curly-2.

An attempt to obtain intra-H1 recombinants between non cross-reacting a, and g-complex antigens was unsuccessful, probably because the high degree of heterogeneity between these alleles prevents intragenic recombination (Yamaguchi and Iino, 1969). In the H2 alleles, only a preliminary study of intragenic recombination between H2 - 1,2 and H2 - e,n,x has been reported (Iino, 1960).

Mapping of the sites in the flagellin gene responsible for flagellar shape has also been carried out. By transductions between a phase-1 curly mutant and fla$^-$ forms of i antigenic mutants of S. typhimurium, Joys and Stocker (1963) mapped the curly mutant site between two mutant sites for antigenicity. In three curly mutants of g-complex antigenic type, the mutant sites have been mapped outside of the cluster of the determinants of antigen factors in H1 (Horiguchi et al., 1975).

It has also been reported that, not only in Salmonella, but also in B. subtilis, flagellar shape mutants are not accompanied by any serological alteration (Iino, 1962a; Iino and Mitani, 1967a; Martinez et al., 1968). These results support the assumption that the key region in flagellin determining flagellar shape is distinct from those determining antigenic specificities.

Sensitivity to flagellotropic phage is also controlled by the flagellin genes. Chi phage of Salmonella (Edwards and Meynell, 1967), phage PBS1 of B. subtilis (Joys, 1965), phage ϕx_7 of the Proteus providence group (Appelbaum et al., 1971), and phage 7-7-1 of Rhizobium lupini H13-3 (Lotz et al., 1977) are all examples of bacteriophage which can attack only flagellate-motile bacteria. In the chi phage-E. coli system, Schade and his co-workers (1967) observed that empty phages, that have already injected phage DNA into the host

cells, increased with time at the bases of flagella after phage infection, but not on the flagella themselves; they suggested that the ultimate receptor site for the phage is located at the base of the bacterial flagellum and that a flagellotropic phage slides along the filament of the flagellum to the base. The resistance of a bacterium to the phage is acquired not only by deflagellation, but also by paralysis, either genetic or physiological (Frankel and Joys, 1966; Meynell, 1961). Therefore, the presence of flagella is not enough for the susceptibility of bacteria to the phage; the flagella must be active.

The host range of a flagellotropic phage, chi, has been studied in relation to the specificity of flagella. Chi phage can infect not only various serotypes of Salmonella but also certain strains of Arizona (Meynell, 1961), E. coli and Serratia marcescens (Iino and Mitani, 1967b), suggesting that the flagella of these bacteria have common phage receptor structures. However, Salmonella serotypes with flagellar antigens of the g-complex, l, or e, h type are generally resistant to chi phage, although host-range mutants of chi phage have been obtained which will attack these serotypes (Meynell, 1961; Sasaki, 1962). A host-range mutant, M8, was shown to be adsorbed to the same extent to all of the g-complex antigenic flagella tested. However, there was a difference in M8 sensitivity among these g-complex antigenic strains which was attributed to some factors other than their antigenic characters, factors which control the infection process after adsorption (Yamaguchi, 1967).

b) Phase Variation

In Salmonella, two genes code for the major flagellar structural protein, flagellin. These two genes, the H1 and H2 genes, map in different regions of the Salmonella genome (Lederberg and Edwards, 1953). The phenomenon of phase variation refers to the ability of the

cell to alternate or switch between expression of the two flagellin structural genes. Phase variation was discovered by Andrewes (1922) in S. typhimurium. Later the presence of phase variation in many Salmonella strains was recognised by various workers as reviewed by Kauffman (1964). Phase variation has also been detected in some strains of Arizona (Fife et al., 1960).

The frequency with which cells undergo phase variation varies with different Salmonella strains from 10^{-3} to 10^{-5} per bacterium, per generation (Stocker, 1949). Analysis by transduction (Lederberg and Iino, 1956) showed that the phase of a given bacterium is determined by the "state" of its H2 locus: when H2 is in the active state, the production of the phase-1 flagellin by H1 is repressed, while H2 carries out the production of phase-2 flagellin, and when H2 changes to the inactive state the production of phase-1 flagellin, specified by H1, proceeds (Iino, 1969a). As for the expression of each H gene, a closely linked factor ah was found to be responsible for activation of the adjoining H gene. Mutation of $ah1^{+}$ to $ah1^{-}$ or $ah2^{+}$ to $ah2^{-}$ results in the failure of production of phase-1 or phase-2 flagellin, respectively (Iino, 1961c; 1962b). An active H2 gene prevents expression not only of a chromosomal H1 allele but also of an H1 allele forming part of an abortively transduced chromosome fragment (Pearce and Stocker, 1967): this and other evidence indicates that the H2 locus controls expression of H1 by determining production of an H1-repressor substance. There is now good evidence (Fujita, Yamaguchi and Iino, 1973) that the repressor substance is distinct from the flagellin specified by gene H2, and is coded for by another gene, termed rhl. When hybrid λ vehicles carrying the H2 and rhl genes were used to program protein synthesis in U.V.-irradiated cells, the synthesis of a 16,000 molecular weight polypeptide was correlated with rhl gene product activity (Silverman, Zieg and Simon, 1978). The repressor gene, rhl, thus constitutes an operon

with H2 and ah2. Ah2 controls the activity of both H2 and rhl, and is regarded as the operator region. In the $ah2^-$ mutants the abilities of both phase-2 flagellin synthesis and inhibition of phase-1 flagellin synthesis are lost. Consequently the phenotype of such mutants becomes stable phase 1 (Iino, 1969a; Fujita, Yamaguchi and Iino, 1973). However, rhl remains active in phase 2 of the $H2^-$ mutants, and neither phase-1 nor phase-2 flagellins are synthesised in this phase. Thus the mutant clones reveal H-O variation. In the rhl^- mutants, synthesis of both phase-1 and phase-2 flagellin proceeds in phase 2 and flagellar filaments are composed of both types of flagellin. In phase 1, they produce only phase-1 flagellin.

To elucidate the step blocked by the phase 1-repressor in phase 2, a cell-free system for in vitro protein synthesis was set up (Suzuki and Iino, 1973). RNA derived from Salmonella was used to direct the protein synthesis; chromatographic analysis of the products showed the presence of flagellin characteristic of the flagellar antigenic type of the cells from which the RNA was extracted. Thus, when RNA was extracted from the cells of the diphasic strain propagated from a single colony expressing either phase 1 or phase 2, the in vitro synthesised flagellin was predominantly the same as that produced by the original colony. Translocation of mRNA specific for phase-1 flagellin was not inhibited by the presence of mRNA specific for phase 2. This experimental evidence thus supports the hypothesis that phase variation is due to the alternative synthesis of phase-specific mRNA, and that the phase-1 repressor blocks the transcription of the ahl-Hl operon.

The repression of the ahl-Hl operon by rhl is not complete throughout the growth phases of diphasic bacteria. Temporary derepression of phase-1 flagellar formation occurs in a fraction of the phase-2 cell population at late exponential and early stationary phase when a cell generation time exceeded 80 minutes (Iino, Oguchi and

Hirano, 1975). The resulting flagellar filaments contain copolymer segments of phase-1 and phase-2 flagellins. The duration of detectable copolymer formation in a cell is of the same order as the half-life of flagellin mRNA (Suzuki, Enomoto and Hirota, 1974). Thus it seems the flagellum-forming apparatus of each flagellum is phase non-specific, being able to incorporate newly synthesised phase-1 flagellin even after it has already started filament formation in phase 2.

To explain the alternative activation and inactivation of the ah2-H2-rh1 operon, the presence of a phase determinant was proposed. Experiments on expression of H1 and H2 in H2 abortive transductants have shown that a phase determinant controls the expression only of the flagellin-specifying gene cis to it, and is without effect on the activity or inactivity of another H2 flagellin-specifying gene, in trans (Pearce and Stocker, 1967). Investigations of the monophasic S. abortusequi (Iino, 1961a) indicated that the the instability of the phase determinant is not intrinsic but is under the control of a chromosomal factor termed vh2. The vh2 factor is closely linked to H2 but separable from it. Replacement of $vh2^+$ in a diphasic clone by $vh2^-$ of a monophasic clone causes the stabilisation of the state below 10^{-7} per bacterial division in its existing state. On the basis of these findings, Iino (1969a) proposed various hypotheses on the mechanism of regulation of the H2 state by the vh2-ah2 system.

Recently, molecular cloning techniques have been used to isolate the segment of Salmonella DNA which contains the H2 region attached to phage λ (Zieg et al., 1977). Heteroduplex analyses revealed an anomaly in the cloned fragment, that is, an apparent inversion of a 900 bp region, which was shown to be adjacent to the H2 gene. A correlation was demonstrated between the phase state of the H2 gene and the sequence of the adjacent segment. It was therefore proposed that an inversion of DNA adjacent to H2 is responsible for the flagellar

phase variation in Salmonella. A deletion, which removed about 50% of the DNA sequences on one side of the invertible region, was found to fix the H2 gene in the H2 (on) state (Silverman et al., 1979). To further define the functions involved in the inversion process, Silverman and Simon (1980) isolated switching mutants defective in the phase transition process. The mutants defined a region of DNA inside the inversion region termed hin, which codes for a gene whose product is necessary for the inversion process.

Destabilisation of the H2 state when the H2 region of the phase-2 stable Salmonella is incorporated in the E. coli chromosome may also be associated with some sort of structural anomaly of the chromosomal region (Enomoto and Stocker, 1975).

c) Fla genes

Other flagella genes involved in flagellar formation are given the gene symbol, fla, followed by a capital letter and/or a Roman numeral designating a cistron (Iino, 1969a). These genes were first disclosed by studies of nonmotile mutants of diphasic Salmonella strains (Stocker, Zinder and Lederberg, 1953). The characteristic of fla is that a mutation of any of the fla genes from fla^+ to fla^- results in the loss of the ability to produce flagella in both phase 1 and phase 2. Mutants of S. typhimurium were classified into eight complementation groups, namely the cistrons fla A, B, C, D, E, F, J and K (Iino and Enomoto, 1962; 1966; Joys and Stocker, 1965). A fla^- mutant of S. paratyphi B was also found to belong to group A (Iino and Enomoto, 1966; Joys and Stocker, 1965), and among 11 fla^- mutants of S. abortusequi, 10 were assigned as fla A, C or F. The remaining one was distinct from any cistrons detected in S. typhimurium and was given the symbol fla G (Iino and Enomoto, 1966). Strains carrying fla G are characterised by the production of flagellin which is antigenically indistinguishable from normal flagellin (Iino and Enomoto, 1966; Iino

and Haruna, 1960), and which can be incorporated into flagellar fibres in an *in vitro* reconstitution process (Suzuki and Iino, 1966). Cistrons fla A, B, C, D, E, J and K were found to be co-transduced with H1 by P22 phage; that is, they are closely linked to H1 (Enomoto, 1967; Iino and Enomoto, 1966; Joys and Stocker, 1965). Iino and Enomoto (1962) termed these H1-linked fla cistrons the fla I group.

Later five additional cistrons were detected among fla⁻ mutants of *Salmonella* strains: fla L (Yamaguchi and Iino, 1967; 1969); fla M (Vary and Stocker, 1973); fla N, fla P and fla Q (Yamaguchi et al., 1972). The three latter cistrons were detected in *S. abortusequi*, but not in *S. typhimurium*.

Of these five cistrons, only fla M was not co-transduced with H1 although it is located near the structural gene. The chromosomal location of fla F, the only other cistron not co-transducible with H1, was mapped by colicin-mediated conjugation between the gal and trp operons (Smith and Stocker, 1962). Complementation tests divided fla A mutants into three subgroups, fla AI, fla AII and fla AIII (Yamaguchi et al., 1972). In addition, some paralysed mutants were found to belong to the same group as non-flagellate fla AII mutants and were located at one end of the fla AII gene.

The presence of fla genes was also demonstrated in *E. coli*. These fla genes are located near H which is homologous to H1 of *Salmonella*, and also between trp and gal (Armstrong and Adler, 1969a). In subsequent studies, large numbers of chi-resistant mutant strains were ordered into complementation groups, by the use of F factors (Silverman and Simon, 1973a). Further genetic analysis with strains carrying mu phage-induced mutations defined additional fla cistrons and revealed the transcriptional organisation of the flagellar genes (Silverman and Simon, 1973b). Thus the flagellar mutants were assigned to three regions of the *E. coli* chromosome. Region I is located between trp and

and gal, region II between uvrc and aro D, and region III between his and uvrc. In region III, fla A, fla P, fla Q and fla R are all cotranscribed. Fla B, fla C, fla O and fla E also form a cotranscribed unit. Later, Komeda ~~et~~ et al., (1980) identified the flb C gene (flb is equivalent to fla) located between hag and fla N.

In region II are located fla I, fla G and fla H, and, in addition, the mot and che genes which will be discussed in the next section. Recently, the flb B gene was found to be part of an operon: flb B - fla I (Komeda et al., 1980). Fla I also has associated with it a region called cfs. This appears to be a regulatory region that makes the synthesis of fla I dependent upon the presence of cAMP. The fla I product was clearly involved in the positive control of the entire flagellar gene complex (Silverman and Simon, 1974b). However, this polypeptide and the polypeptides corresponding to the fla G and fla H genes have not been identified.

Initially, only three genes, fla K, fla L and fla M, that formed a cotranscribed unit, were described for region I (Hilmen, Silverman and Simon, 1974). However, more recent complementation tests suggest that there are more genes in this region. Thus far, at least six have been identified (Komeda et al., 1980).

Comparison of the E. coli and Salmonella genetic maps (Figure 3) reveal a great deal of homology in the distribution of flagellar genes. Intergeneric complementation tests by P1-mediated transduction from E. coli donors to S. typhimurium recipients revealed that most of the fla gene products were interchangeable (Kutsake et al., 1980). However, Salmonella appeared to have gene functions that were not found in E. coli. For example, nml, the gene responsible for the methylation of flagellin, was present in Salmonella, but not in E. coli (Stocker, McDonough and Ambler, 1961; Konno et al., 1976). In addition, there is a region in Salmonella that has no homologue in E. coli, i.e. the phase

FIGURE 3 Linkage map of the flagella genes of *Salmonella* (S) and *E. coli* (E).

his: histidine operon including A-I cistrons.

trp: tryptophan operon including A-E cistrons.

tre: trehalose; arg: arginine; aro: aromatic amino acids;

pyr: pyrimidine; pur: purine: uvr: ultraviolet light sensitivity;

nml: N-methyl lysine in flagellin; hag, H1, H2: flagellin;

mot: motility; che: chemotaxis.

Other symbols on which only cistron designations are given are fla (flagellation).

An arrow indicates the direction of transcription of an operon.

A dotted line indicates a pair of homologous cistrons.

The chromosomal position of each linkage group is shown as "min" in the 138 min linkage map of *S. typhimurium*.

The arrangement of the genes in parentheses to their outside genes is not known.

Figure taken from review by Iino (1977).

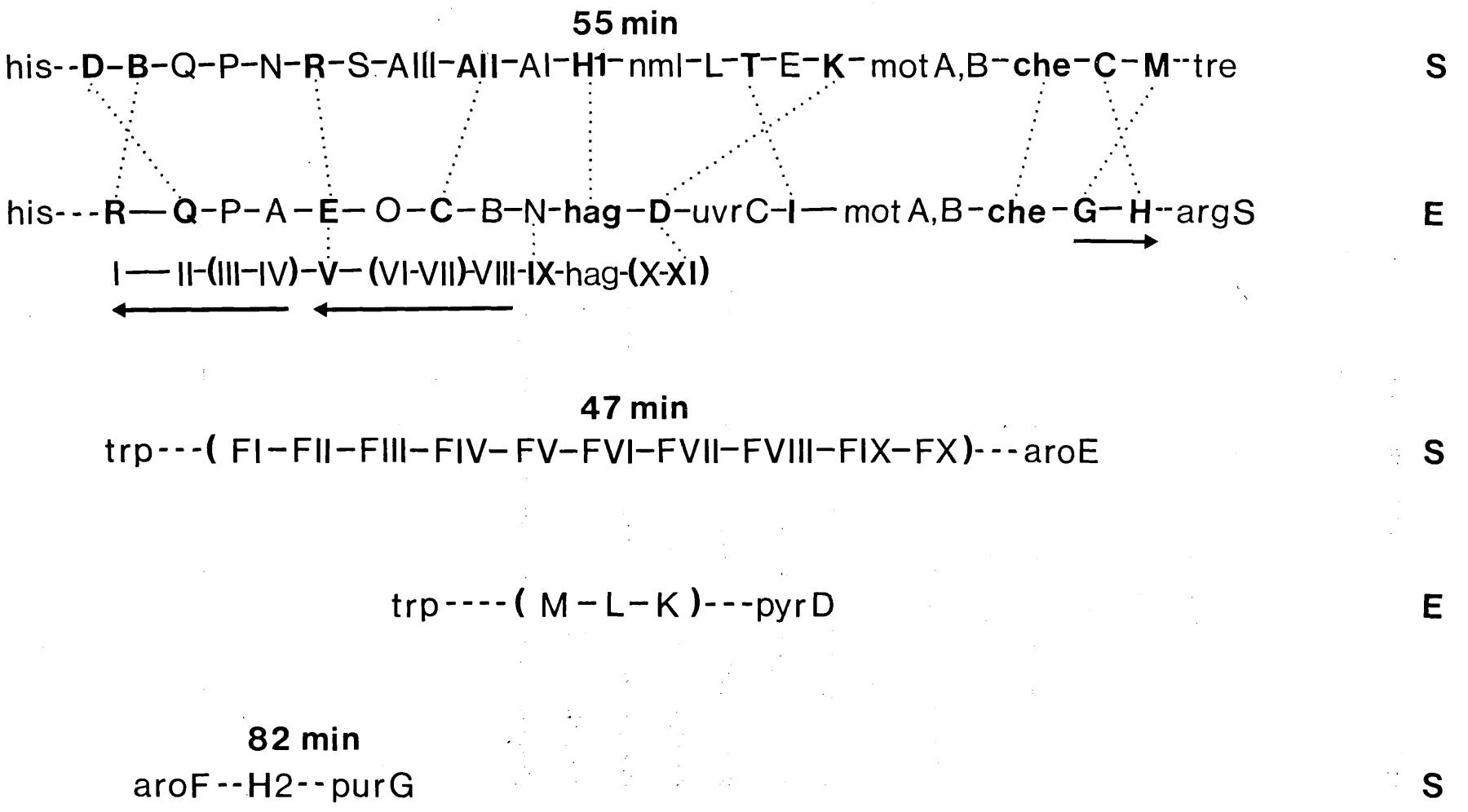

55 min
his--D-B-Q-P-N-R-S-AIII-AII-AI-H1-nml-L-T-E-K-motA,B-che-C-M--tre
S
his---R—Q-P-A-E-O-C-B-N-hag-D-uvrC-I—motA,B-che-G-H--argS
E
I—II-(III-IV)-V-(VI-VII)-VIII-IX-hag-(X-XI)
47 min
trp---(FI-FII-FIII-FIV-FV-FVI-FVII-FVIII-FIX-FX)---aroE
S
trp----(M-L-K)---pyrD
E
82 min
aroF--H2--purG
S

Figure 3

2 region, which is located between purC and strA (Mäkelä, 1964; Smith and Stocker, 1962).

No advance has been made on mapping of the flagella genes in B. subtilis since Joys and Frankel (1967) reported three nonallelic genes H, fla and mot, with transformation experiments. On P. mirabilis, eight fla and one mot gene were disclosed (Appelbaum and Prozesky, 1973). Except for one fla, they were located in a cluster transduced by phase 34·13 simultaneously. Flagella genes of Ps. aeruginosa were also mapped in a cluster in which at least one hag, ten fla and one mot were detected by phage F116-mediated transduction (Iino T, unpublished information - cited in Iino, 1977). In contrast, the fla genes of Caulobacter crescentus were not mapped in a cluster (Johnson and Ely, 1978). In addition, three mot loci were mapped, as compared to two in E. coli and Salmonella.

d) mot genes

Among the non-motile mutants, paralysed mutants have been studied the most intensively in Salmonella, next to nonflagellate (fla^-) mutants. Paralysed mutants are non-motile, although they produce flagella indistinguishable from those of the motile parent. Extensive studies were carried out on nearly 100 paralysed mutants from S. typhimurium by means of transduction (Enomoto, 1966a) and sexual recombination (Enomoto, 1966b). The paralysed mutants were classified into three cistrons: mot A, mot B and mot C. Mot A and mot B are jointly transduced by a single P22 phage particle, while mot C is transduced with Hl. Mot C was later found to be identical with fla AII (Yamaguchi et al., 1972). Interestingly, a mutation in this cistron results in a defect of flagellar formation or paralysis of flagella depending on the site of mutation.

In E. coli two cistrons, mot A and mot B responsible for motility of flagella, were disclosed (Armstrong and Adler, 1967; Silverman,

Matsumura and Simon, 1976). Mot A and mot B, together with che A (discussed in next section) are organised into an operon termed Mocha (Silverman and Simon, 1976). Molecular cloning techniques were used to identify the specific proteins corresponding to the two mot genes (Silverman, Matsumura and Simon, 1976; Silverman and Simon, 1976). The molecular weights for mot A and mot B are 31,000 and 39,000, respectively. As intracistronic weak complementation occurs in many combinations of mot A^- and mot B^- mutants (Enomoto, 1966a; 1966b; Armstrong and Adler, 1967), it is possible that the product proteins of these mot cistrons function as a structural complex. A plausible function of these proteins is that they participate in the link of the rotary apparatus of a basal body with the connecting membrane structure, or with the machinery supplying energy for the rotation.

e) che genes

Che genes were initially described by Armstrong and Adler (1969a) and subsequently by Parkinson (1976) in E. coli, and by Vary and Stocker (1973) and Aswad and Koshland (1975) in Salmonella. Strains of bacteria carrying these mutations have distinctive phenotypes; they either swim smoothly without changing direction or they tumble incessantly (Parkinson, 1974). Che^- mutants are presumably defective in their ability to integrate input information and to transmit it to the flagellar organelle (Parkinson, 1975). Analysis of the complementation behaviour of the Che^- mutants that map near the mot gene in E. coli was carried out by Armstrong and Adler (1969b) and by Parkinson (1976). They concluded that there were two genes, che A and che B (Armstrong and Adler, 1969b) and that each showed extensive intracistronic complementation (Parkinson, 1976). It is now clear that the gene formerly referred to as che A is made up of two genes, che A and che W (Silverman and Simon, 1977b).

The che A gene directed the synthesis of two polypeptides with

apparent molecular weights of 66,000 and 76,000. Peptide map analysis of the two polypeptides indicated that they have most of their peptides in common. Furthermore, measurements of the coding capacity of the DNA that corresponded to the che A gene indicated that it was sufficient to code for a single polypeptide of 76,000 molecular weight. It was therefore proposed that the smaller polypeptide was derived from the larger one either by proteolysis or by the presence of a second initiation point within the che A gene. The che W gene product has an apparent molecular weight of 12,000 (Silverman and Simon, 1976; 1977b).

The che M gene is adjacent to the che W gene and codes for the synthesis of a group of polypeptides that appeared on sodium dodecyl sulphate (SDS) polyacrylamide gels as three bands of apparent molecular weights 63,000, 61,000 and 60,000 (Silverman et al., 1977). This "triplet" protein is a major component of the inner membrane of the cell and appears to be the same as MCP (methyl-accepting chemotaxis protein). MCP has been identified as a membrane protein which is methylated reversibly; its methylation is related to chemotaxis (Kort et al., 1975). However, analysis of the genetic basis of the formation of MCP indicated that these polypeptides were the products of two different chemotaxis genes, che M and che D (Silverman and Simon, cited in Silverman and Simon, 1977a). The product of the che D gene appears as a group of bands with molecular weights corresponding to 64,000 and 65,000. Thus the MCP polypeptides range from approximately 60,000 to 65,000 molecular weight.

Adjacent to the che M gene is the che B complex. This is composed of four genes, che X, che B, che Y and che Z: the product of the che X gene had a molecular weight of 28,000; the che B gene product had a molecular weight of 38,000, whereas che Y, which is adjacent to che B, synthesised a polypeptide with a molecular weight of 8,000; and che Z was responsible for the synthesis of a 24,000 molecular weight

polypeptide. All four of these genes may be cotranscribed, beginning with che X and reading through che Z (Silverman and Simon, 1977b). The products of the che X, che B, che Y and che Z genes, as well as those of che A and che W, seem to be located in the cytoplasm, although a transient association with the inner membrane cannot be excluded (Ridgeway, Silverman and Simon, 1977).

In Salmonella, chemotaxis mutants were isolated and mapped between mot B and fla C, as well as within the fla AII gene (Collins and Stocker, 1976). More recently, the use of preformed vertical gradients of chemoattractants (Aswad and Koshland, 1975) enabled the isolation of a large number of Che$^-$ mutants which have been divided into 9 complementation groups (Warrick, Taylor and Koshland, 1977). Of these, 5 map at the end of the flagella region, 2, che U and che V, map in the fla Q and fla A II genes respectively, and the remaining genes, che S and che T, have not yet been mapped.

4. Chemical Modification of Proteins

Chemical modification of proteins is performed for a wide variety of reasons including identification of residues involved in catalysis and binding, and detection of conformational change. A general treatment of the subject is given in a review by Cohen (1968).

In the case of the bacterial flagellar protein, flagellin, chemical modification has mainly been used to limit the sites of trypsin-catalysed hydrolysis to arginyl residues in order to facilitate the determination of amino acid sequences, to solubilise large peptides, to investigate the hydrophobic nature of intermolecular bonds in flagella and to distinguish surface resides from those in the interior of the molecule.

a) Amino groups

Chemical modification of amino groups has mainly been used to limit the sites of trypsin-catalysed hydrolysis of proteins to arginyl

residues in the determination of amino acid sequences. In the early sixties, carbon disulphide (Merigan et al., 1962), ethylthiol trifluoroacetate, (Goldberger and Anfinsen, 1962) and methyl acetimidate (Hunter and Ludwig, 1962) were introduced for the reversible blocking of amino groups. However, the usefulness of all these reagents in facilitating sequence analysis has been very limited. The introduction in 1967 of maleic anhydride for the reversible blocking of amino groups represented a major advance (Butler et al., 1967; 1969). Maleic anhydride reacts with amino groups to yield maleyl derivatives that are stable in alkaline and neutral solution but are labile at acid pH. Following the introduction of maleic anhydride, many other acid anhydrides, for example, 2-methyl maleic (citraconic anhydride and 2, 3-dimethylmaleic anhydride (Dixon and Perham, 1968) were brought into use. Maleic anhydride, however, is not absolutely specific for amino groups. O-Maleyl derivatives of tyrosyl, seryl and threonyl residues are formed in small amounts (Butler et al., 1969). This may cause certain arginyl bands, located close to the carboxyl groups of such maleylated residues, to become resistant to tryptic digestion (Brattin and Smith, 1971). Slow tryptic hydrolysis of arginyl and lysyl bands with adjacent acidic residues has been observed in many proteins.

Reversible modification with maleic anhydride has been used to limit tryptic digestion to arginine residues in the sequence studies of B. subtilis flagellin (Shaper et al., 1976). However, maleylation has also been very useful for other purposes. For example, the 3 relatively insoluble peptide fragments produced by cyanogen bromide digestion of P. mirabilis flagellin were maleylated (Glossman and Bode 1972). This treatment rendered them soluble, allowing subsequent separation by gel filtration.

The charge changes brought about by maleylation can be made the

basis of diagonal electrophoretic methods for examining amino acid sequences around amino and thio groups (Tang and Hartley, 1967; 1970), and, in favourable cases, help to establish the rôle of amino groups in macromolecular structure.

b) Arginine residues

Procedures for the modification of arginine residues in proteins are based upon their reactions with aldehydes and with 1, 2- or 1, 3-dicarbonyl compounds. The aldehyde adducts are labile while condensation with dicarbonyl compounds leads to the formation of relatively stable heterocyclic rings. The subject has been reviewed by Yankeelov (1972). A selective modification of arginine and of arginyl residues can be achieved with 1, 2-cyclohexanedione in sodium borate buffer at pH 8 to 9 over the temperature range 25° to 40°C. A single condensation product is obtained under these mild conditions (Patthy and Smith, 1975a). The product is stable both in borate buffers (pH 8 to 9) and in acidic solutions. Regeneration of arginyl residues is attained readily by incubation in hydroxylamine buffers at pH 7.0. Patthy and Smith (1975a; b) have reported successful applications of this reaction in amino acid sequence analysis, and in structure-function studies.

Arginine residues may also be modified with glyoxal (Kotaki et al., 1968) and phenylglyoxal (Takahashi, 1968). The exact structure of the derivatives is not known. According to Takahashi (1968) the derivative formed with phenylglyoxal is stable at acid pH, but arginine is slowly regenerated in the absence of excess reagent at neutral or alkaline pH. Glyoxal and phenylglyoxal also react with α-amino groups to yield the α-keto derivative. This latter reaction can be used to identify the end groups of small peptides by subtractive analysis (Takahashi, 1968). To overcome the problem of the side reaction with amino groups, Freedman et al. (1968) first maleylated the protein to be modified,

thus restricting modification with glyoxal to arginine residues.

c) Methionine residues

The thioether side chain of methionine residues at acid pH can be selectively modified by performic acid, α-haloacids and amides, β-propiolactone and alkyl halides. Performic acid converts methionine to the sulphone which is readily quantitated by amino acid analysis. This reaction was used by Parish and Ada (1969) to obtain a more accurate estimate of the methionine content of S. adelaide flagellin.

Chemical modifieation of methionine residues has also been used to investigate the hydrophobic nature of intermolecular bonds in B. pumilis flagellin (Smith and Koffler, 1971). As methionine residues are thought to reside primarily in hydrophobic regions, it was surmised that modification of these residues with iodoacetic acid to form the carboxymethyl sulphonium salt should decrease the hydrophobic nature of methionine-containing regions. In the intact filament, methionine residues are not exposed to the reagent, since carboxymethylation does not occur at pH 5.5 to 6.5. At pH 2.2, carboxymethylation of methionine residues follows pseudo first order kinetics (Smith and Koffler, 1968). All residues react and do so at the same rate. Destruction of the ability to reassemble during carboxymethylation also follows pseudo first order kinetics. These data indicate that association of flagellin molecules to form the filament structure is sensitive to alterations in hydrophobic regions of the molecule. Ichiki and Martinez (1968) reported that treatment of B. subtilis flagellin with sodium periodate completely destroyed the ability to reassemble, although they did not comment on the possible mechanism involved. Likewise, flagellin of B. pumilis fails to reassemble following treatment with periodate (Smith and Koffler, cited in Smith and Koffler, 1971). These latter workers suggested that the failure of modified flagellin to reassemble was due to oxidation of the methionine

residues.

d) Tyrosine residues

The UV absorption spectra of proteins above 270nm can be accounted for almost exclusively by the content of tyrosine and tryptophan. The absorbance of tryptophyl residues is independent of pH, while tyrosine ionisation results in a large red shift and an increase in absorbance of this chromophore.

Modification of the phenolic ring of tyrosine results in the generation of chromophores with distinctive absorption maxima and widely different extinction coefficients. The pKa of the phenolic hydroxyl is likewise sensitive to substitutions on the ring. Consequently, modification of tyrosyl residues usually may be monitored by spectrophotometry. Unfortunately, side reactions involving other residues are frequently not detectable by this technique.

Acetylimidazole, N-Bromosuccinimide, cyanuric fluoride and diazonium compounds are commonly used reagents for the chemical modification of tyrosyl residues, but most extensive use has been made of iodination and nitration.

Iodination of proteins at mildly alkaline pH results in the formation of mono- and di-iodo derivatives of tyrosine (Glazer and Sanger, 1964). However, iodination of histidine and oxidation of cysteine and methionine side chains may also occur. A particularly interesting development has been the introduction of milder iodination procedures employing lactoperoxidase as catalyst (Morrison, 1968). Iodination of native proteins has been used in attempts to distinguish surface tyrosine residues from those in the interior of the molecule. To determine the site in S. adelaide flagellin where iodination preferentially occurred, Parish and Ada (1969) iodinated flagellin (monomer) and polymer (flagellin purified by polymerisation in 15% saturated ammonium sulphate) with a mixture of (^{125}I) iodide and

carrier iodide. Each preparation was then treated with cyanogen bromide (CNBr). At small extents of substitution, the tyrosine residue of CNBr fragment D of flagellin was more readily substituted than those of CNBr fragment A. By contrast, in polymerised flagellin, the tyrosine residues of fragment A were more readily substituted. These differences in the patterns of iodination of flagellin and of polymer were interpreted in the following way: in the polymer, fragment A is exposed, but fragment D is masked. This is of particular interest as fragment A contains 10 of the 11 ϵ-N-methyllysine residues of the molecule; methylation of lysine is enzymatically mediated (Kim and Paik, 1965). Thus, Parish and Ada put forward the theory that methylation of flagellin lysine residues may occur only after polymerisation of the flagellin.

Since introduction by Sokolovsky et al. (1966), tetranitromethane (TNM) has been widely used for the modification of tyrosine residues in proteins (Riordan and Sokolovsky, 1971). The pKa of 3-nitrotyrosine is approximately 7, and the nitrophenoxide ion absorbs intensely in the visible region of the spectrum. This simultaneously permits evaluation of the degree of nitration and assessment of the effect of the drastic change in the pKa of the modified residue(s) upon structure and function. 3 nitrotyrosine is fairly stable to acid hydrolysis and can be quantitated by amino acid analysis.

Although the usefulness of TNM in studies of tyrosine modification is unquestionable, an awareness of the problems involved in its reaction with proteins is essential. TNM promotes inter- and intramolecular cross-linking in proteins and, in some instances, this can be the dominant reaction. Although some cross-links undoubtedly involve bonds between tyrosine side chains, other types of cross-links may also be formed (Shifrin and Solis, 1972). In addition to cross-linking, TNM may also oxidise cysteinyl, methionyl and tryptophyl

residues. However, in spite of these limitations, modern techniques for the fractionation and characterisation of closely related macromolecules are good enough to permit isolation of homogenous protein derivatives. The resolution of the products of nitration of P. mirabilis flagellin is a good example (Schalch and Bode, 1975). Tyrosine residues were modified with TNM to ascertain their involvement in the protomer-protomer interaction of P. mirabilis flagella. Analysis of the flagellin-TNM reaction mixture by SDS polyacrylamide gel electrophoresis revealed the presence of both monomeric and dimeric nitrated flagellin species. The monomeric nitrated flagellin was separated from the dimeric species by gel filtration and subjected to isoelectric focussing in order to detect other heterogeneities and side reactions upon nitration. In this way, a nitrated flagellin species was obtained which was homogenous in respect to molecular weight and charge. Aggregation experiments showed that this highly purified monomeric nitroflagellin had completely lost its aggregation ability due to the incorporation of the nitro group. In contrast to the isolated native flagellin, none of the 5 tyrosines in the polymeric flagellum were nitrated by TNM. Thus, it was concluded that the integrity of the phenolic groups is necessary for the proper folding and aggregation of the flagellin subunits to form the stable helical flagella.

Intermolecular and intramolecular cross-linking during nitration with TNM has also been reported for B. stearothermophilus flagellin (Smith and Koffler, 1971). When monomeric flagellin is modified at pH 8.5, two derivatives can be separated, one being the monomer with 3 of the tyrosine residues modified. The other is a dimer apparently covalently linked through 2 modified tyrosine residues, one furnished by each monomer. Not only do the isolated monomers assemble into filaments, but so do the purified dimers, suggesting that tyrosine-

tyrosine interactions may be involved in assembly of flagellin subunits to form the flagella.

The aim of the project was to obtain a more detailed knowledge of the primary structure of bacterial flagellins in order to investigate the following:

1) The amino acid sequence basis for H antigenic variation in Salmonella g.... antigenic flagellins, and the location of the antigenic sites of the flagellin molecule.

2) The sites of amino acid substitutions resulting from mutations which cause changes in flagellar filament shape and function, thus enabling the conformation and bonding of subunits in the flagella filament to be better understood.

3) The presence of ε-N-methyllysine residues in certain bacterial flagellins, and their location in the flagellin molecule.

4) Whether certain amino acids are buried or exposed on the surface when flagellin molecules are polymerised to form the flagella filament.

5) Phylogenetic relationships between bacteria by comparison of amino acid sequences of flagellins, assuming that conserved sequences may indicate regions essential for self-assembly.

MATERIALS AND METHODS

A. MATERIALS

1. Bacterial cultures

All *Salmonella* strains were monophasic.

The *Salmonella* strains, H1-antigen g, s, t (SL 736), g, m (SL 166), f, g (SL 175) and b (SL 174) were from the collection of Professor B.A.D. Stocker, formerly of the Lister Institute of Preventive Medicine, now at the Department of Medical Microbiology, Stanford University, California, U.S.A. Other *Salmonella* strains carrying g .... antigens, g, t, z_8, z_9 (5724), m, t (5743), g, m, s (5747), g, q (10480), g, p, u (5767) and g, m, t (6018) were from the National Collection of Type Cultures (N.C.T.C.), Central Public Health Laboratory, Colindale Ave., London, NW9, as were *Pseudomonas fluorescens* (10038) and the *Proteus* strains: *P. morganii* 10041, 2815 and 232; *P. mirabilis* 6197; *P. rettgerri* 7475; *P. vulgaris* 4175 and 10020; *P. inconstans* 6344. Other *Proteus* strains were a gift from Professor Coetzee at the University of Pretoria, South Africa. *Erwinia carotovora* was from the Botany Department, Imperial College, London SW7, England; *Escherichia coli* K12 and *Bacillus subtilis* 168 from Professor W. Hayes, originally for genetic teaching purposes; all other strains from the National Collection of Industrial Bacteria (N.C.I.B.), Torry Research Station, Aberdeen.

Morphological mutants of *Salmonella* g ... antigenic strains were isolated by Dr. M.W. McDonough and Mr. D. Hogben, both of the Department of Botany, Bedford College, Regent's Park, London NW1, England, and are preceded by the letter M or D, respectively.

2. Chemical reagents

Chemical reagents used were of the "Analar" grade unless otherwise stated; their source, for example, Sigma Chemical Company, B.D.H. Chemicals Ltd., etc., is given with description of method.

B. METHODS

Stock cultures

Stock cultures of bacteria were maintained on agar slopes and regularly sub-cultured to maintain viability.

1. Production and Isolation of Flagella

To obtain highly flagellated, and thus motile, organisms, bacteria from the stock cultures were allowed to swarm from a central inoculum over nutrient agar: 0.5% NBA was used for selecting motile organisms of the *Proteus* strains, while motile *Salmonella* cells were selected on Edward's semi-solid agar:-

Difco Bactopeptone	10g
Gelatin	80g
Agar	3.5g
NaCl	5g
Distilled water	1 litre

The medium was steamed until the gelatin was thoroughly dissolved, dispensed into medical flats, and autoclaved at 15 lbs/sq. inch steam pressure for 15 minutes.

Nutrient broth (Lab. M, 2.5%) was inoculated with motile organisms from the outside edge of the swarm, incubated overnight at 37°C, and then assessed for motility using a x 40 objective. These highly flagellated bacteria were then grown on an enriched agar medium consisting of:-

Tryptone	60g
Casein Hydrolysate	30g
Yeast Extract	30g
Glycerol	60ml
Sodium lactate	30ml
Lab M Agar	100g
Distilled water	8 litre

The medium was autoclaved at 15 lbs/sq. inch steam pressure for 30 minutes, cooled, distributed evenly between 20 sterile, lidded, stainless steel trays 25cm x 30cm x 5cm and any bubbles present removed by passing a bunsen burner flame over the surface. The agar was then allowed to set. A few ml of the inoculated broth were distributed over the surface of each tray of enriched agar with a sterile glass spreader, and the trays were incubated at 37°C for 18 hours or at 25°C for 40 hours.

The bacteria were harvested by adding about 20ml of 0.85% NaCl to each tray, and scraping off the growth from the surface of the agar with a glass spreader. The suspension was blended in a M.S.E. blender to remove the flagella from the bacterial cells.

2. Preparation of flagella and flagellin

The bacteria were separated from the flagella by centrifugation in a M.S.E. 18 high speed centrifuge (Measuring and Scientific Equipment) at 10,000 x g (9,000 r.p.m.) for 30 minutes at 10°C. The supernatant was removed and centrifuged at 35,000 x g (17,500 r.p.m.) for 1 hour at 10°C to give a clear, gelatinous pellet of flagella. A further spin yielded another, smaller pellet of flagella. The pellets of flagella were suspended in distilled water.

Flagella were either stored frozen or freezedried.

To prepare a flagellin solution, the suspension of flagella was diluted with distilled water, and then adjusted to pH 2.0 by the dropwise addition of 1M HCl, with stirring. The acid-insoluble material was removed by centrifuging at 35,000 x g for 30 minutes at 10°C, and the clear supernatant obtained brought to pH 6.0 by the dropwise addition of 1M NaOH.

3. Reaggregation of flagellin

The flagellin was reaggregated by the addition of solid $(NH_4)_2SO_4$

to give a 70% saturated solution. The flocculant white precipitate obtained was sedimented by centrifugation at 35,000 x g for 30 minutes, dissolved up in a small volume of distilled water, and dialysed at 4° against several changes of distilled water and finally against deionised water. To further purify the polymer, centrifugation at 10,000 x g for 30 minutes to sediment any unwanted material, followed by centrifugation at 35,000 x g for 1 hour was employed. Purified flagellin was obtained by redissociation of this pellet.

4. Freezedrying

A suitable volume of the sample (not more than about one-fifth of the volume of the flask) was placed in a 100ml or 250ml round-bottomed flask. The sample was frozen by rotating the flask in a solid CO_2-acetone mixture until an even layer was deposited around the glass. The flask was then attached by a cone on a straight stem to the freezedrier (Birchover Instruments Ltd.) which had been allowed to reach a vacuum of 100mT or less. Alternatively, the sample was allowed to freeze after the flask had been attached to the freezedrier, although it was necessary not to allow loss of the sample due to excessive frothing prior to freezing.

The samples were usually freezedried overnight.

5. Polyacrylamide gel electrophoresis

a) Determination of homogeneity of flagellins

The method was carried out according to Parish et al (1969) at pH 2.7 in 6M urea using a Shandon Analytical Polyacrylamide Gel Electrophoresis Apparatus (1SAE2734).

The following solutions were prepared:

Acrylamide

30g acrylamide + 0.8g N,N'-methylenebisacrylamide, made up to 100ml with water.

Gel Buffer

7.5mM KOH, 0.58M Acetic acid, pH 2.7

2.52g KOH + 204ml Glacial acetic acid, made up to 1 litre with water.

Ammonium persulphate

1.5g in 100ml water, freshly prepared.

Reservoir Buffer

0.42g KOH + 34ml Glacial acetic acid, made up to 1 litre with water.

Gel Stain

1.25g Coomassie brilliant blue dissolved in 454ml 50% methanol, followed by addition of 46ml Glacial acetic acid and filtration.

Destaining Solution

50ml methanol + 75ml Glacial acetic acid, made up to 1 litre with water.

(i) Preparation of gels

In most experiments, 10% gels were used. They were prepared using the solutions described above, by mixing together:

4.0g	Urea
2.0ml	Gel buffer
1.8ml	water
4.2ml	Acrylamide
0.4ml	Ammonium persulphate

0.08ml of N, N, N', N'-Tetramethylethylene diamine ("TEMED") was added as an initiating agent for polymerisation. (The quantities given are sufficient for 8 tubes.)

The gel solution was dispensed into siliconised glass tubes of inside diameter 5mm, length 8-10cm, which were closed by Silicone-rubber caps at one end, filling the tubes to within 1cm of the top. The tubes were tapped to remove air bubbles, and water carefully layered on top of the gel solution. The gels were allowed to set at 37°C for 30 - 45 minutes. Just before use the water layer was sucked off, the caps removed, and the tubes placed in the electrophoresis apparatus.

(ii) Preparation of samples

30 - 150µg of freezedried material or one drop (0.04ml) of protein solution were mixed with one drop of 8M urea, and one drop of 50% glycerol containing methyl green (tracking dye). If necessary, the sample was diluted with gel buffer.

(iii) Electrophoresis

The prepared protein samples were applied to the top of the gels, and reservoir buffer carefully layered on top of each sample to fill the tubes. Electrophoresis was carried out using gel buffer diluted 1:6 as the reservoir buffer, the anode being at the top of the apparatus. Using a Shandon Vokam constant voltage/constant current D.C. power supply to deliver 3mA per gel, electrophoresis took about 2 hours to be completed, as indicated by the position of the marker band.

(iv) Staining

The gels were removed from the glass tubes by injecting water from a syringe between gel and glass wall, and pushing the gel out with the aid of a rubber teat; they were stained in Coomassie blue for 30 - 60 minutes. Bands could normally be detected within an hour or two of destaining, but complete removal of background stain took several days and required several changes of destaining solution.

b) Molecular weight estimations of flagellins

Molecular weights were estimated by means of the sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis technique using the system of Laemmli (1970). The system can also be used to characterise proteins and check for homogeneity in the absence of SDS.

The following solutions were prepared:

Acrylamide

30% acrylamide, 0.8% n, N'-methylenebisacrylamide, aqueous.

Ammonium persulphate

1.5%, aqueous, freshly prepared.

Gel Buffer

1M Tris-HCl buffer, pH 8.8.

SDS

1% or 10%, aqueous.

TEMED

50%, aqueous.

Reservoir Buffer

0.025M Tris, 0.192M glycine, pH 8.3

3g Tris, 14.4g glycine and 1g SDS, made up to 1 litre with water.

(i) Preparation of gels

In most experiments, 12.5% gels were used. They were prepared by mixing:

10.0ml	Gel buffer
1.8ml	Water
11.0ml	Acrylamide
2.7ml	1% SDS
1.2ml	Ammonium persulphate

0.04ml of 50% TEMED was added to initiate polymerisation.

The gel solution was dispensed into glass tubes as described previously, and allowed to set at room temperature for 30 minutes.

(ii) Preparation of samples

30 - 130µg freezedried material or 1 drop of protein solution were mixed with one drop of each of the following solutions: gel buffer, pH 8.8; 10% SDS; 0.05% bromophenol blue (tracking dye) and 50% glycerol. In the case of the reference proteins, a drop of mercaptoethanol was also added to the mixture. The samples, and reference proteins, were then heated at 95°C for 2-3 minutes, cooled, and applied to the gels. Electrophoresis was carried out as described above; the anode at the bottom of the apparatus. Before the gels were stained, a small piece of thin wire was inserted in each gel to mark the position of the dye front.

The following proteins were used as molecular weight standards:

Bovine serum albumin (66,000)

Catalase (60,000)

Egg albumin (ovalbumin) (45,000)

Trypsinogen - bovine pancreas - treated with phenylmethylsulphonyl fluoride (24,000)

Cytochrome c (11,700)

Catalase and cytochrome c were obtained from Boehringer Mannheim, W. Germany; other proteins from Sigma Chemical Company, Poole, Dorset, U.K.

(iii) Estimation of molecular weights

Proteins of known molecular weight (reference proteins) were included in each experiment, and their mobilities were calculated from the relation:

$$\text{Mobility} = \frac{\text{Distance protein migrated}}{\text{Distance dye migrated}}$$

A standard curve of mobility vs. the logarithm of the molecular weight of these proteins was used to estimate the molecular weights of unknown proteins from their calculated mobilities (Figure 1·7).

6. Amino acid compositions of flagellins.

a) Complete acid hydrolysis

1mg of protein in 0.5ml 6N HCl was hydrolysed in a glass tube, 15 x 0.75cm, with a partly drawn out neck. After evacuating the tube with a water pump, the neck was drawn out and sealed in a flame. The samples were incubated at 105-110°C for 16 hours, then either dried *in vacuo* over P_2O_5 and NaOH pellets or in a rotary evaporater.

b) Quantitative amino acid analysis

Evaporated hydrolysates were dissolved in 1ml of pH 2.2 citrate buffer and diluted further if necessary. 0.8ml aliquots were then injected into the sample loops (0.5ml capacity) of an automatic amino

acid analyser (LKB Instruments Model 4101). The cation exchange column was eluted sequentially by the following sodium citrate buffers: buffer A, 0.2N, pH 3.5 (10 minutes); buffer B, 0.2N, pH 4.25 (40 minutes); buffer C, 1.2N, pH 6.25 (1 hour 24 minutes). The temperature of the column was increased from 50° to 70°C, 40 minutes after the start of the run. After 10 minutes regeneration time with 0.4M NaOH, the column was re-equilibrated with buffer A (45 minutes), the whole procedure taking a total of 3 hours. Resolution of ∈-N-methyl-lysine (NML) was obtained by replacing buffer C with 0.35N, pH 5.28 buffer, thereby increasing the total elution time by 1.5 hours. Authentic N-∈-methyl-L-lysine HCl was obtained from Bachem Inc., California, USA.

A standard mixture of amino acids (LKB or Sigma), containing 25 nmoles of each amino acid, was analysed at regular intervals to calibrate the amino acid analyser. (Figure 4).

b) Qualitative amino acid analysis

The amino acid composition of hydrolysates was analysed qualitatively by either paper electrophoresis at pH 3.5, or a combination of chromotography and paper electrophoresis at pH 1.9. In the latter case, the hydrolysed sample was spotted onto Whatman Grade 2 Chroma paper (measuring 20 x 40cms), 2cm from a corner at the anode end. At pH 1.9, the amino acids are positively charged and move towards the cathode. However, at pH 3.5 the acidic amino acids are negatively charged and move towards the anode, thus the origin is positioned 15cms from the anode end, the sample being applied as a 5cm streak (Figure 5).

The buffers used for electrophoresis:

	pH 1.9	pH 3.5
Pyridine	-	1ml
Formic acid	15ml	-
Glacial acetic acid	5ml	10ml
Water	80ml	90ml

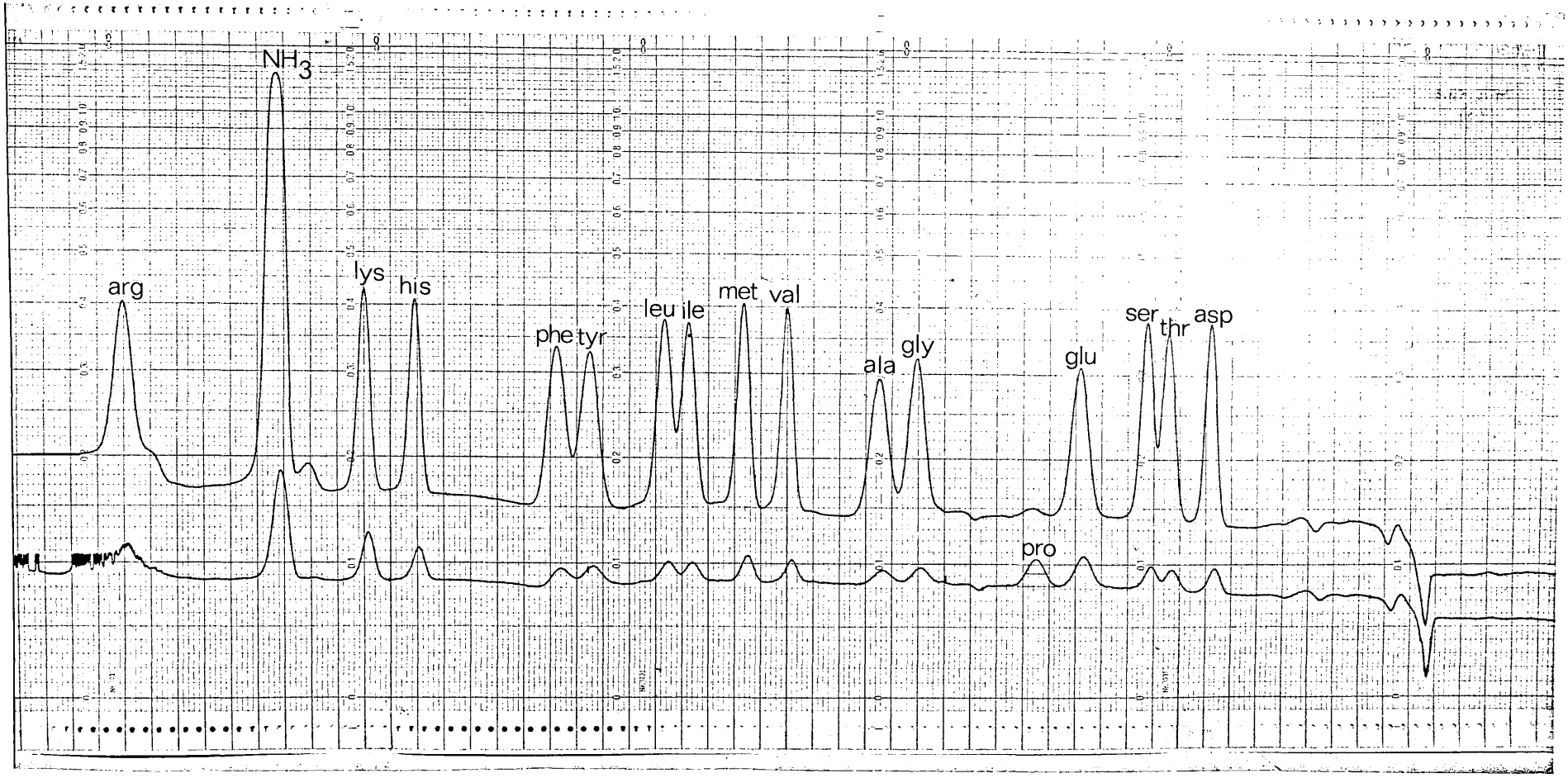


Figure 4 Analysis of a standard mixture of amino acids

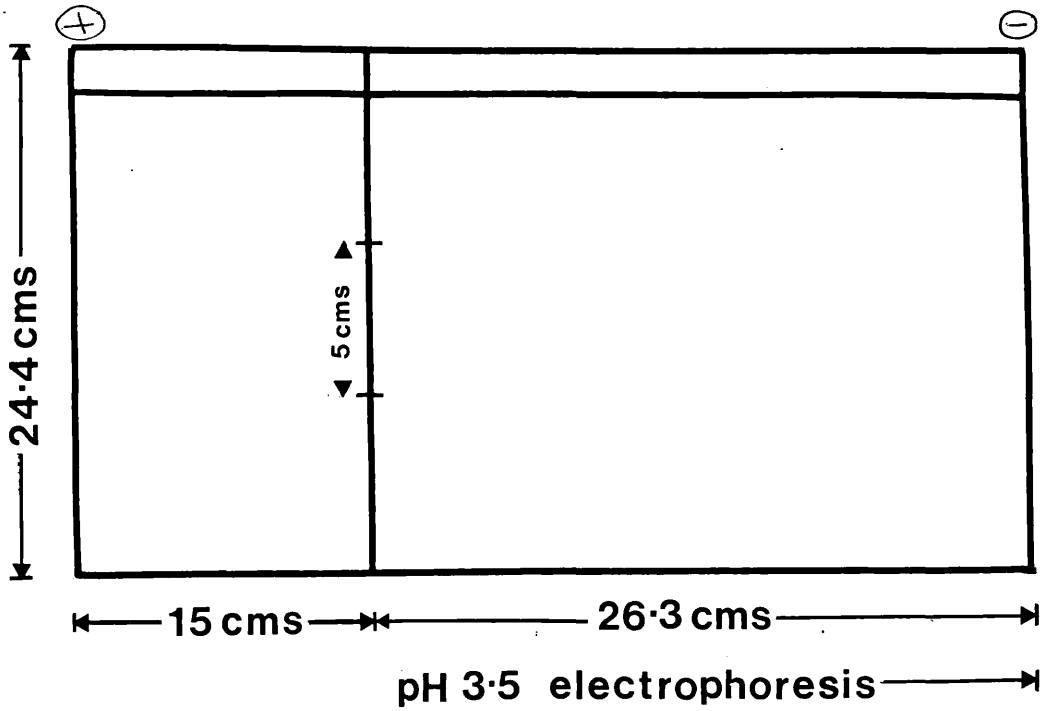


Figure 5

The paper was carefully wetted with the appropriate buffer, using a pipette, and electrophoresis was carried out in a flat bed, high voltage electrophoresis apparatus (Camag, Muttenz, Schweiz) at 2,500 volts for 40 minutes.

Where necessary, the chromatogram was thoroughly air dried, rolled into a cylinder and subjected to ascending chromatography in a Shandon Unikit tank. The solvent used was the upper phase of a mixture of butan-1-ol:glacial acetic acid:water (4:1:5 v/v). The paper was removed, air dried and dipped in a solution of 0.2% ninhydrin (w/v) in acetone. When dry, the paper was heated carefully over a wire gauze covered flame until spots began to appear.

7. Cyanogen bromide cleavage of flagellin

Cyanogen bromide specifically and almost quantitatively cleaves peptide chains on the carboxyl side of the methionine residues, converting methionine into homoserine lactone (Figure 6). Thus a protein with three methionine residues should produce four fragments on reaction with cyanogen bromide. This reaction is therefore particularly useful in sequence and other studies as it can potentially provide intermediate sized fragments of the protein.

The whole procedure was carried out in a fume cupboard due to the extremely poisonous nature of the reagent.

An approximately 100-fold excess (w/w) of cyanogen bromide (Sigma Chemical Company, Poole, Dorset, U.K.) over methionine residues was added to a flagellar solution of about 100mg in 5ml 70% formic acid. The reaction was allowed to proceed in a stoppered round-bottomed flask overnight at room temperature. The reaction mixture was then diluted 1:10 with distilled water and freezedried to remove excess cyanogen bromide. The only satisfactory solvent for freezedried cyanogen bromide digests, which did not cause aggregation of the fragments, was 98-100% formic acid.

FIGURE 6

Cleavage of a polypeptide chain at a methionine residue by cyanogen bromide.

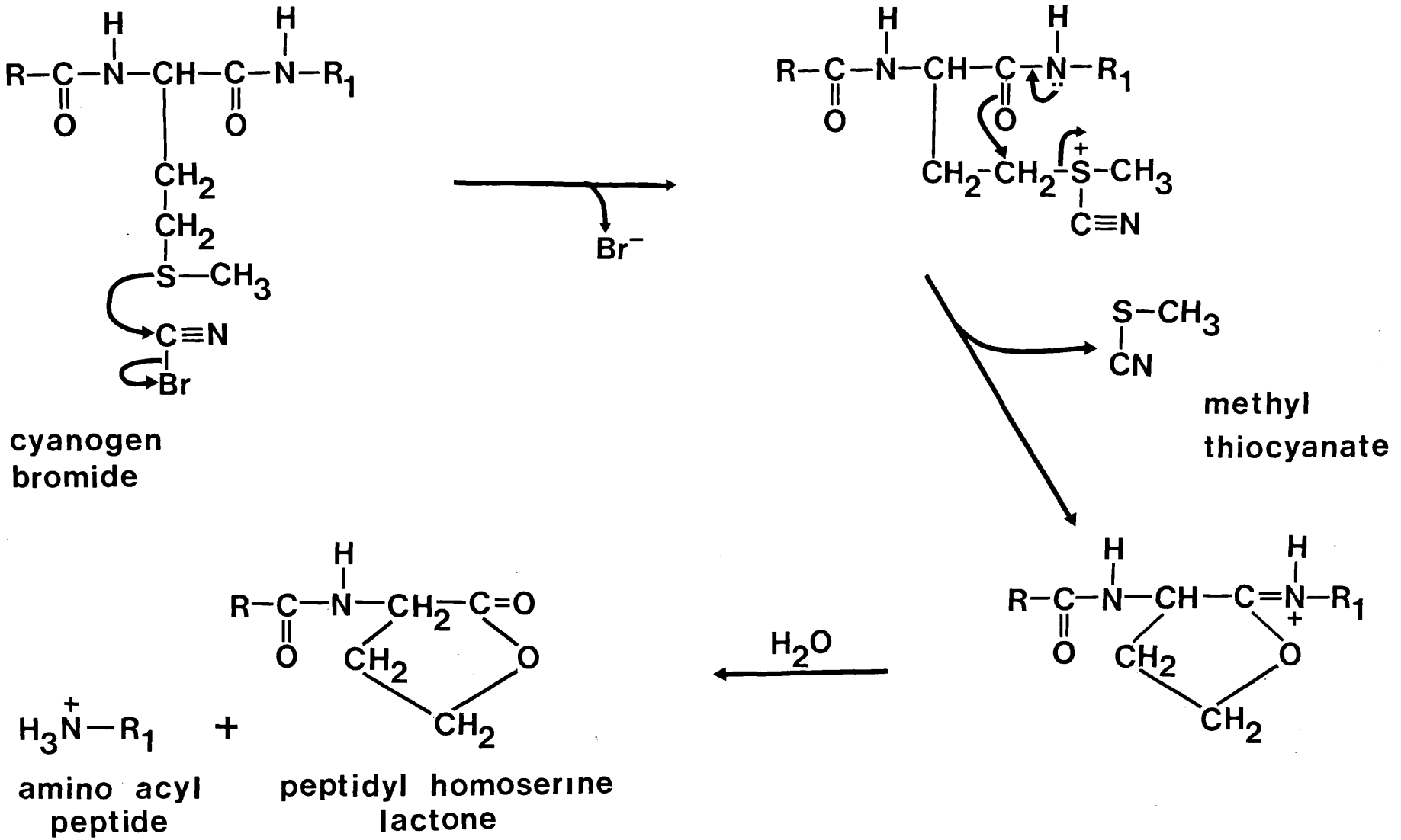

cyanogen bromide
Br⁻
methyl thiocyanate
H₂O
amino acyl peptide
peptidyl homoserine lactone

Figure 6

8. Gel filtration of cyanogen bromide digests

100mg freezedried cyanogen bromide digest were dissolved in 10ml of 98-100% formic acid and applied to a freshly poured 100cm x 2.5cm column of Sephadex G75 (40-120μ) (Pharmacia, Uppsala, Sweden), previously equilibrated with 70% formic acid. The column was eluted with 5% formic acid at room temperature. The flow rate was approximately 20ml per hour; 4ml fractions were collected in an LKB Ultrorac fraction collector, using the drop counting mechanism. The fractions were monitored by polyacrylamide gel electrophoresis, pH 2.7 system, and the appropriate fractions were pooled and freezedried. Additional refractionation on Sephadex G75 (40-120μ) or G-50 (Fine) columns was employed to further purify fractions.

Alternative methods were sometimes used to monitor fractions, for example, the measurement of extinction at 215nm, after removal of formic acid by dialysis for 2 hours (Parish and Ada, 1969), or the measurement of turbidity $\overset{\alpha}{\phi}t$ 420nm after the addition of 6% trichloroacetic acid (Garrick et al, 1978).

9. Chloramine-T-Oxidation of Flagellin

The method was carried out according to Parish and Ada (1969) with some slight modifications. 30mg of freezedried, polymerised flagella were dissolved in 2ml of 0.12M NaCl - 0.02M sodium phosphate buffer, pH 7.3, dissociated into flagellin with 0.15ml of 1M HCl, and then neutralised with 0.15ml of 1M NaOH. To this solution was added 7.5mg of chloramine-T (BDH Chemicals Ltd., Poole, Dorset, England) dissolved in 1.75ml of the same buffer. Oxidation was carried out for 9 minutes at room temperature, the reaction being stopped by the addition of 0.75ml of potassium metabisulphite (Harrington Brothers Ltd., London) (20mg/ml). The reaction mixture was dialysed overnight at 4°C against distilled water and then freezedried, a small sample being kept for polyacrylamide gel electrophoresis. The freezedried material was

redissolved in 2ml of 70% formic acid, digested with cyanogen bromide and fractionated by gel filtration, as described above.

10. Enzymic Digestion

a) Complete enzyme digestion

(i) Trypsin and Chymotrypsin

1mg of freezedried sample was dissolved in 0.1ml of 0.05M NH_4HCO_3, heated in a waterbath at 95°C for 30 seconds and rapidly cooled under running tap water. Trypsin (Diphenyl carbamyl chloride-treated) or chymotrypsin was added at an enzyme to substrate ratio of 1:50 (w/w). Digestion was allowed to proceed for 1.5 hours at 37°C. Any precipitate present was removed by centrifugation at 6,000 x g using an M.S.E. bench centrifuge.

(ii) Thermolysin

1mg of freezedried sample was dissolved in 0.1ml of 0.05M NH_4HCO_3, and thermolysin added at an enzyme to substrate ratio of 1:25 (w/w). Digestion was allowed to proceed for 1.5 hours at 37°C, or for 1 hour at 50°C.

(iii) Pepsin

1mg of freezedried sample was dissolved in 0.1ml of 5% formic acid (pH 2.0), and pepsin added at an enzyme to substrate ratio of 1:25 (w/w). Digestion was allowed to proceed for 1 hour at 37°C.

(iv) Staphylococcal protease (V8)

1mg of freezedried sample was dissolved in 0.1ml of 0.05M NH_4HCO_3, pH 7.8, and staphylococcal protease added at an enzyme to substrate ratio of 1:100 (w/w). The reaction mixture was incubated at 37°C for 2 hours.

(v) Papain

1mg of freezedried sample was dissolved in 0.1ml distilled water, and a small amount of papain suspension added. The reaction mixture was incubated at 37°C for 1.5 hours.

(vi) Carboxypeptidase A and B

Carboxypeptidase A was prepared immediately before use by washing the crystals (suspended in water with toluene added) by centrifugation to remove contaminating amino acids, and then dissolving in 10% (w/v) lithium chloride).

Both carboxypeptidase A and B were diisopropylphosphofluoridate (DFP) - treated to inhibit the activity of other pancreatic proteases, expecially chymotrypsin. Protein samples were prepared for digestion by dissolving 2mg of freezedried material in 0.5ml of 0.05M NH_4HCO_3, heating at 95°C for 30 seconds, and then dialysing overnight at 4°C against 0.005M NH_4HCO_3 to remove extraneous amino acids. Carboxypeptidase A and/or B was added in the ratio of approximately 1:50 (w/w) of enzyme to substrate, and allowed to react at 37°C. Samples were taken at timed intervals and diluted into cold pH 2.2 citrate buffer to stop the reaction. Subsequently, the samples were subjected to quantitative amino acid analysis.

Trypsin, thermolysin (B. thermoproteolyticus Rokko), pepsin (hog stomach), papain (papaya latex), carboxypeptidase A (bovine pancreas) and carboxypeptidase B (hog pancreas) were obtained from Sigma Chemical Company, Poole, Dorset, U.K.; chymotrypsin from Worthington Biochemical Corporation, Freehold, N. Jersey; Staphylococcal protease (S. aureus) from Miles Laboratories Ltd., Stoke Poges, Buckinghamshire, U.K.

b) Partial enzyme digestion

(i) Trypsin

The method described by Ichiki and Parish (1972) was used.

Freezedried protein material was digested with trypsin (1µg/mg protein) for 24 hours at 25°C in 0.05M borate buffer, pH 8.0.

(ii) Pepsin

The method of Ichiki and Parish (1972) was used. 0.5M sodium acetate buffer, pH 5.0 was added to the protein solution until the

concentration of acetate was 0.1M. Pepsin dissolved in water (10mg/ml) was added to give a final concentration of 2% w/w of enzyme. Digestion was performed at 37°C for 2.5 hours.

c) Isolation of tryptic cores

Trypsin digests of whole flagella or cyanogen bromide fragment A were prepared as described in 10a)(i) above. Tryptic peptides were removed by dialysis against distilled water at 4°C leaving an undialysable tryptic core. The latter was freezedried and characterised by polyacrylamide gel electrophoresis at pH 2.7.

11. Peptide Analysis

a) Ion-exchange Chromatography

The method used was that of Schroeder (1967). The freezedried trypsin digest, dissolved in 0.2M pyridine acetate buffer, pH 3.1 and centrifuged to remove any insoluble material, was applied to a 1cm x 100cm column of Dowex AG50-X2 cation exchange resin (Biorad Laboratories, Richmond, California, USA), previously equilibrated with 0.2M pyridine acetate buffer, pH 3.1.

Peptides were eluted with a buffer gradient of increasing concentration and pH prepared by pumping 666ml of 2.0M pyridine acetate, pH 5.0 buffer at a constant rate into a mixing chamber containing 333ml of 0.2M pyridine acetate pH 3.1 buffer. 1.5 - 2ml fractions were collected in an LKB Ultrorac fraction collector, using the drop counting mechanism.

The following buffer gradient systems were also used in order to resolve particular mixtures of peptides.

(i) 250ml 0.425M pyridine acetate, pH 3.7
500ml 1.625M pyridine acetate, pH 4.6

(ii) 250ml 0.6M pyridine acetate, pH 4.0
500ml 1.2M pyridine acetate, pH 4.6

(iii) Combination of two buffer gradient systems:

1. 200ml 0.2M pyridine acetate, pH 3.1

400ml 0.8M pyridine acetate, pH 4.6

Followed by:-

2. 170ml 0.8M pyridine acetate, pH 4.6

340ml 1.6M pyridine acetate, pH 4.6

Peptides were detected with ninhydrin after alkaline hydrolysis of samples (Moore and Stein, 1954). To aliquots of 0.1ml - 0.3ml of each fraction, 1ml of 2.5N NaOH was added, and the tubes heated in a covered boiling water bath for 1.5 hours. After cooling, the contents of each tube were neutralised by the addition of 1ml of 30% acetic acid, and then 1ml of ninhydrin reagent was added to each.

Ninhydrin reagent:

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

The tubes were heated in a boiling water bath for exactly 15 minutes, cooled, and 2ml 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 570nm. The absorbance values were plotted against tube number and significant peaks pooled, concentrated in the rotary evaporator, and characterised by high voltage paper electrophoresis at pH 6.5. If a peak contained more than one peptide, these peptides were purified by a combination of paper electrophoresis at pH 6.5 and paper chromatography in the upper phase of a mixture of butan-1-ol:glacial acetic acid:water (4:1:5 v/v).

b) Peptide analysis on paper

(i) Peptide maps

Digests were spotted in 5µl amounts onto Whatman 3MM chromatography paper prepared as shown in Figure 7. As indicated, the origin was moved

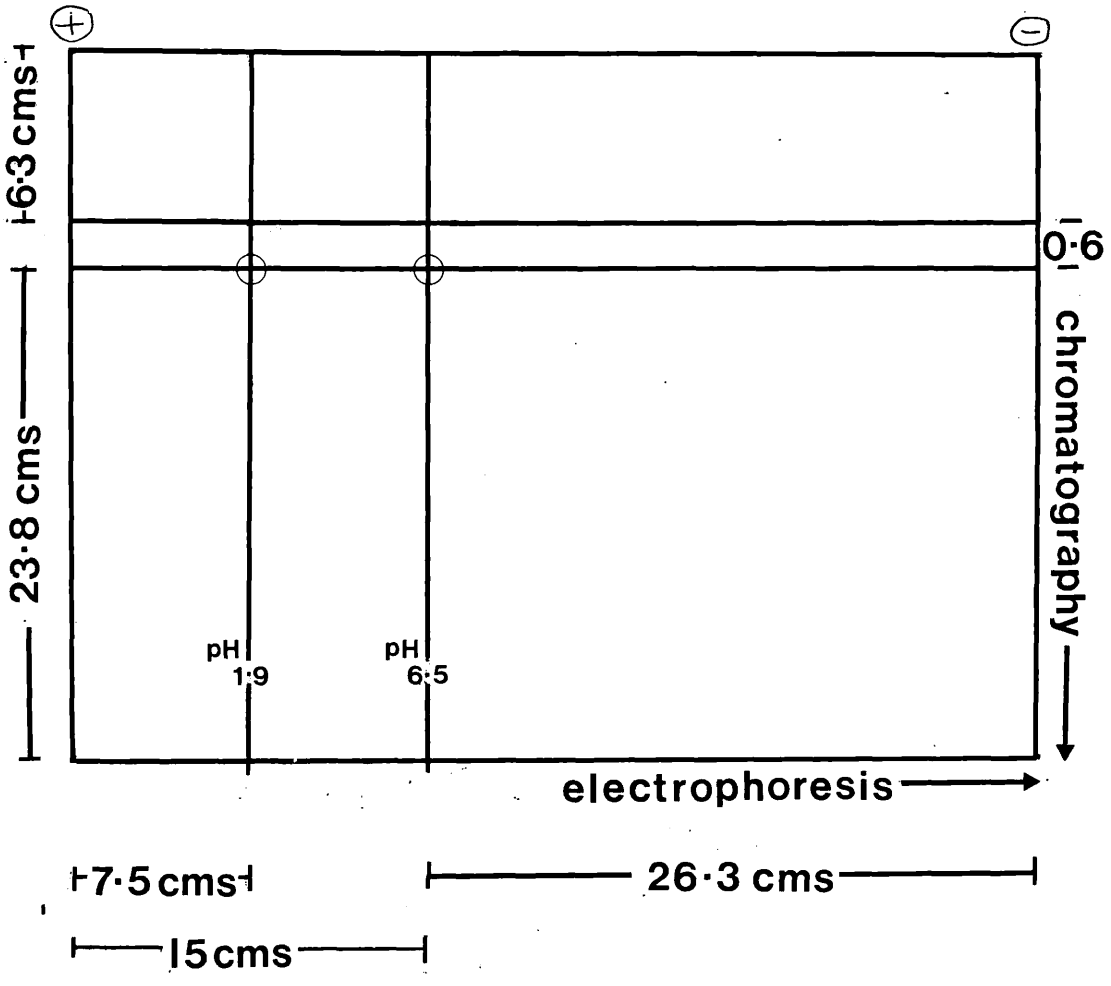

⊕
⊖
6·3 cms
0·6
23·8 cms
chromatography
pH 1·9
pH 6·5
electrophoresis
7·5 cms
26·3 cms
15 cms

Figure 7

closer to the anode when electrophoresis was run at pH 1.9. All peptides are positively charged at this pH, and will migrate towards the cathode. Descending chromatography was carried out for 16-17 hours in a covered Panglass Shandon 500 Chromatank at room temperature. The solvent used was the upper phase of a mixture of butan-1-ol: glacial acetic acid: water (4:1:5 v/v). The maps were left to air dry for 24 hours and were then subjected to electrophoresis in a flat bed electrophoresis apparatus (Camag) at 2,000 volts for 1 hour 15 minutes. Electrophoresis buffers used were:

pH 6.5 Pyridine: glacial acetic acid: water (10:1:90 v/v)

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

When dry, the maps were dipped in 0.2% ninhydrin in acetone, dried, and heated carefully over a wire gauze covered flame until spots began to appear. Any yellow spots (probably indicating N terminal glycine or amide residues) were marked at this stage. The chromatograms were then allowed to develop for 24 hours in the dark.

(ii) Reagents for the identification of specific amino acids present in peptides.

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

Tyrosine

The peptide map was firstly stained with buffered ninhydrin (1ml pyridine, 1ml glacial acetic acid and 98ml 0.3% ninhydrin in acetone) to locate peptides. After marking the spots with a pencil, the ninhydrin stain was removed by dipping the map in 1% HCl in acetone and drying.

Staining solutions:

Solution A 0.1% (w/v) α-nitroso-β-naphthol in acetone.

Solution B 10ml conc. HNO_3 + 90ml acetone (freshly prepared).

The map, after dipping in Solution A, was thoroughly dried before

being dipped in Solution B. After 5-10 minutes drying, the map was warmed carefully over a wire gauze covered flame.

Tyrosine-containing spots were rose coloured, the background light yellow.

Arginine

Staining solutions:

Solution A 0.2% (w/v) 8 OH-Quinoline in absolute ethanol. (0.2ml 8M urea was added to 100ml Solution A to enhance spots.)

Solution B 0.02ml bromine added to 20ml 5% KOH (aqueous) just before use.

The peptide map was sprayed sparingly several times with Solution A. Great care was taken not to overspray. After drying, the map was sprayed with Solution B and dried. Arginine spots were orange-pink; the colour was stable for several hours. The paper was then dipped in 0.2% ninhydrin in acetone to locate the arginine-containing peptides detected.

Histidine (Pauly reaction)

Staining solutions:

Solution A 4.5mg sulphanilic acid + 5ml conc. HCl, made to 500ml 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).

Solutions A, B and C were chilled separately in an ice bath. 1 part B was added to 2 parts A, and the paper lightly sprayed with this mixture. Then the paper was sprayed very lightly with Solution C until spots appeared without fading. Histidine spots were orange-pink, tyrosine spots tended towards brown. After drying, the paper was dipped in 0.2% ninhydrin in acetone to locate the histidine and/or tyrosine-containing peptides detected.

(iii) Elution of peptides from peptide maps

The method of Bennett (1967) was used with slight modifications.

A peptide map of a 5mg enzyme digest was prepared as described previously. The map was stained lightly with 0.025% ninhydrin in absolute ethanol:2N glacial acetic acid (75:25 v/v), dried, and heated until spots began to appear. The spots were circled with a pencil, numbered, and a tracing of the map made. Each spot was carefully cut out and eluted by immersion in 0.02M acetic acid at 4°C overnight. This procedure was repeated several times until sufficient concentrations of each peptide were obtained for amino acid analysis and sequencing. In some cases, the peptides were eluted directly into 6N HCl, hydrolysed and analysed quantitatively.

(iv) Peptide purification on paper

Peptides were purified by electrophoresis at pH 6.5 and/or chromatography.

The sample was applied as a thin streak 15cm from the anodic end of the paper (Whatman 3MM) and subjected to electrophoresis at 2,000 volts for 1 hour 15 minutes. A marker strip, 1cm wide, was developed with ninhydrin and the areas corresponding to the bands on the strip were cut out and eluted by immersion in 0.02M acetic acid. As each band may represent more than one peptide, the eluted material was then chromatographed in butan-1-ol:glacial acetic acid:water (4:1:5 v/v), a marker strip being used in the same way to locate ninhydrin-positive material. The peptides were eluted as above.

12. Sequence analysis

a) Sequential Edman degradation

Edman's (1950) phenyl isothiocyanate reaction (Figure 8) was used to degrade peptides, producing a sequential series of smaller peptides. Phenylisothiocyanate reacts with the free α-amino groups in dilute alkali to yield the phenylthiocarbamyl peptide. Treatment of this product with anhydrous acid causes cyclization. This results in

FIGURE 8

Sequential Edman degradation of a polypeptide chain.

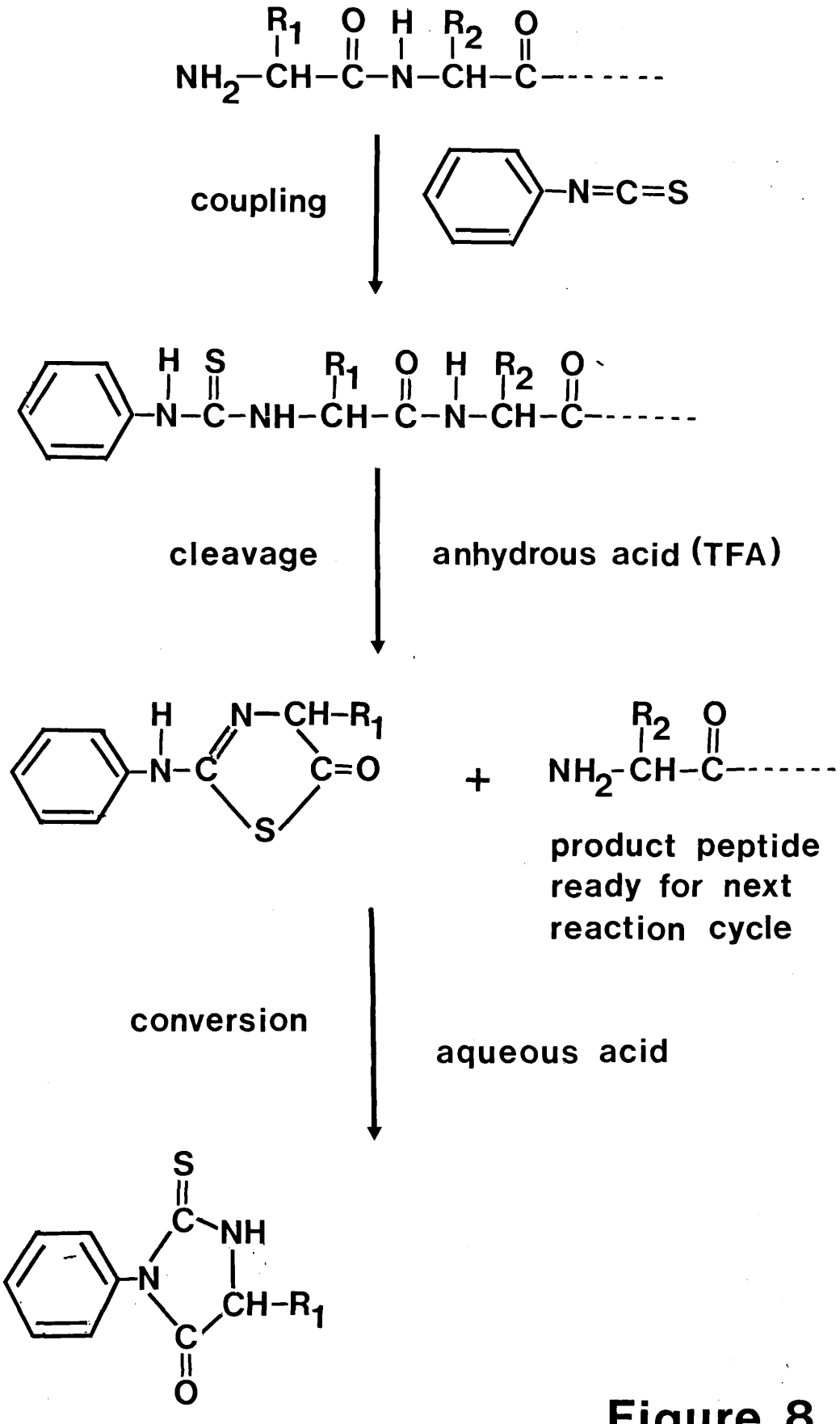

coupling
cleavage
anhydrous acid (TFA)
product peptide ready for next reaction cycle
conversion
aqueous acid
PTH—amino acid

Figure 8

removal of the NH_2-terminal residue as the thiazolinone and the exposure of the α-amino group of the penultimate amino acid. The thiazolinone rearranges to the N-substituted thiohydantoin by heating or by treatment with aqueous acid. Repetition of these reactions results in the stepwise degradation of the peptide. To obtain the amino acid sequence from successive application of these reactions, three principal methods can be used:

1) Direct: the N-substituted thiohydantoins (PTH) can be identified directly by chromatography (Edman and Sjöquist, 1956) or by mass spectroscopy (Richards and Lovins, 1972).

2) Dansyl-Edman: the α-amino group of the residual peptide which is exposed after cyclization can be dansylated (Gray, 1972).

3) Subtractive: a portion of the peptide remaining after cyclization can be taken for hydrolysis and amino acid analysis. Comparison of the amino acid compositions before and after each step should reveal a decrease in the amount of the amino acid that occupied the NH_2-terminal position of the peptide being degraded (Konigsberg, 1972).

The latter two methods were used; in both cases a degradation procedure, suitable when samples were to be degraded through 5-15 steps, was employed:

Solutions used:

Phenylisothiocyanate (PITC) Solution (pure) (Koch-Light Laboratories, Bucks, England).

10% in pyridine, made up fresh weekly. Stored in a glass-stoppered bottle, wrapped in foil, at -10°C. Newly made up solutions were thoroughly flushed with N_2 in a fume cupboard; the bottle was flushed with N_2 every time before being restoppered.

Trifluoroacetic acid (TFA)

Flushed with N_2 before being restoppered.

1,2 Dichloroethane - Analar.

Pyrex test tubes 7cm x 1cm were heat-cleaned at 500°C before use.

Coupling with PITC

The peptide solution was dried in vacuo in a heat-cleaned test tube and dissolved in 200µl of aqueous pyridine (50% v/v, flushed with N_2). An amount of peptide equal to 2-10nmoles per step was used, the larger amounts being taken for longer degradations. When direct identification of the end group using dansylation was to be employed, a sample of suitable size was removed at this stage, and in subsequent rounds, and the original volume restored by the addition of 50% pyridine. To the main sample, 100µl PITC solution was added and the tube flushed with N_2, covered with Parafilm, mixed thoroughly, and left to react for 30 minutes at 50°C.

Removal of excess reagent and pyridine

The sample was dried thoroughly in vacuo at 60°C to remove pyridine, excess PITC and volatile by-products of the reaction. (Diphenylthiourea (DPTU), a non-volatile by-product of the reaction, is removed after cleavage of the PTC-peptide, to avoid extraction losses of the PTC-peptide). At this stage, the tube contents appeared pinkish brown and crystalline. If, however, they appeared oily, they were suspended in 50-100µl of ethanol, and dried again.

Cleavage of PTC-Peptide

The dried residue was dissolved in 50-100µl of anhydrous trifluoroacetic acid (TFA) and the tube flushed with N_2 and sealed with Parafilm. The reaction was allowed to proceed for 10 minutes at 45°C, and the TFA removed in vacuo. After thorough drying, the sample usually adhered as a film to the tube.

Removal of non-volatile by-products

After the cleavage step, the peptide was much less susceptible to extractive losses than was the PTC-peptide. DPTU and the PTC-amino

acid could therefore be removed by extraction with dichloroethane with only a minimal loss of peptide. Water (150µl) was added to the dried residue, which was then extracted using 3 x 1ml amounts of dichloroethane. The two phases were mixed thoroughly with a Vortex mixer, and then separated by centrifugation. The bottom layer, containing DPTU and the PTC-amino acid, was kept in case confirmation of the identity of the cleaved amino acid was required. From the aqueous layer containing the peptide a suitably sized sample was removed either for dansylation or amino acid hydrolysis. The remainder of the peptide was dried and subjected to another cycle of the degradation procedure.

b) Dansyl-Edman degradation

(i) Dansylation

The method of Gray (1967) was used in combination with paper electrophoresis at pH 1.9, or the thin-layer chromatography systems of Woods and Wang (1967).

Peptides

Solutions used:

Dansyl chloride solution (1-Dimethylaminonaphthalene-5-sulphonyl chloride) (Sigma Chemical Company, Poole, Dorset, U.K.).

2.5mg/ml in acetone (analytical grade). The solution was stable at room temperature for several weeks in a vial with a tight-fitting polyethylene cap.

Sodium bicarbonate solution

0.2M in ammonia-free water.

Hydrochloric acid

6N. Analytical grade.

Labelling of peptide

A solution of the peptide (0.5-5nmoles) was transferred to a small glass test tube which had been heat-cleaned at 500°C. After drying *in vacuo*, the peptide was redissolved in 15µl of 0.2M sodium bicarbonate

solution, and dried a second time. This step removed any ammonia present in the sample. The peptide was then dissolved in 15µl of deionised water and about 0.1µl of the solution was spotted onto pH indicator paper. The pH at this point was usually 8.5 - 9.0; if it was below 7.5, more base was added. When the pH was correct, 15µl of dansyl chloride solution was added, the tubes covered with Parafilm and the reaction allowed to proceed for 1 hour at 37°C or 2 hours at room temperature. During this time, most of the excess reagent was hydrolysed to DNS-OH, and the solution became colourless or pale yellow. The solution was dried in vacuo.

Hydrolysis

100µl of 6N HCl was added to the dried residue, the tubes evacuated and immediately sealed in a flame. The tubes were heated for 16 hours at 105°C, then opened and dried down over P_2O_5 and NaOH pellets, in vacuo.

Proteins

Because of the decreased accessibility of the end group to the reagent, dansylation of whole protein requires much more dansyl chloride than is required for peptides.

Solutions used:

Sodium dodecyl sulphate (SDS)

1% aqueous solution (w/v).

N-Ethylmorpholine (BDH Chemicals Ltd., Poole, Dorset, England).

Dansyl chloride solution

25mg/ml in anhydrous dimethyl formamide, freshly prepared.

Labelling of protein

Approximately 1-2mg of protein were placed in a small glass test tube which had been heat-cleaned at 500°C. 200µl of 1% SDS were added and the mixture heated in a boiling water bath for 2-5 minutes to ensure thorough unfolding of the protein. After cooling, 200µl of

N-ethylmorpholine were added, and mixed thoroughly. N-ethylmorpholine acts both as a base and a detergent, and allows reagent to penetrate into insoluble protein particles. The reagent, 300µl of dansyl chloride solution, was then mixed with the dissolved protein and reaction allowed to proceed for 1 hour at room temperature.

Hydrolysis

Approximately 0.5ml of acetone was added to precipitate the labelled protein, and the solutions thoroughly mixed. The precipitate was collected into a pellet by centrifugation, washed once with 500µl of 80% acetone, centrifuged down again, and dried. Hydrolysis was then carried out as described for peptides.

(ii) Identification of dansyl amino acids

Evaporated hydrolysates were extracted with water saturated-ethyl acetate to remove excess DNS-OH and salt. The extracts were dried and dissolved up in acetone-acetic acid (3:2, v/v).

Thin-layer chromatography

The solvent system used was based on that described by Woods and Wang (1967). Polyamide sheets (15cm x 15cm) coated on both sides, were obtained from BDH Chemicals Ltd., Poole, Dorset, England. These sheets were cut into 5cm squares and 2µl samples applied 5mm from one corner using a micropipette. The sample spots were not allowed to exceed 2mm in diameter. Standards containing known amino acids were spotted on the other side of the sheet.

Solvents used:

First solvent	Water : 90% formic acid (200:3, v/v)
Second solvent	Toluene : acetic acid (9:1, v/v)
Third solvent	Butyl acetate : methanol : acetic acid (30:20:1, v/v/v)

Chromatography was carried out in screw-capped glass jars.

FIGURE 9

Separation of a standard mixture of dansyl amino acids by thin-layer chromatography according to the method of Woods and Wang (1967).

Solvent 1 = Water - 90% Formic acid (200:3, v/v)

Solvent 2 = Toluene - Glacial acetic acid (9:1, v/v)

O = DNS-OH

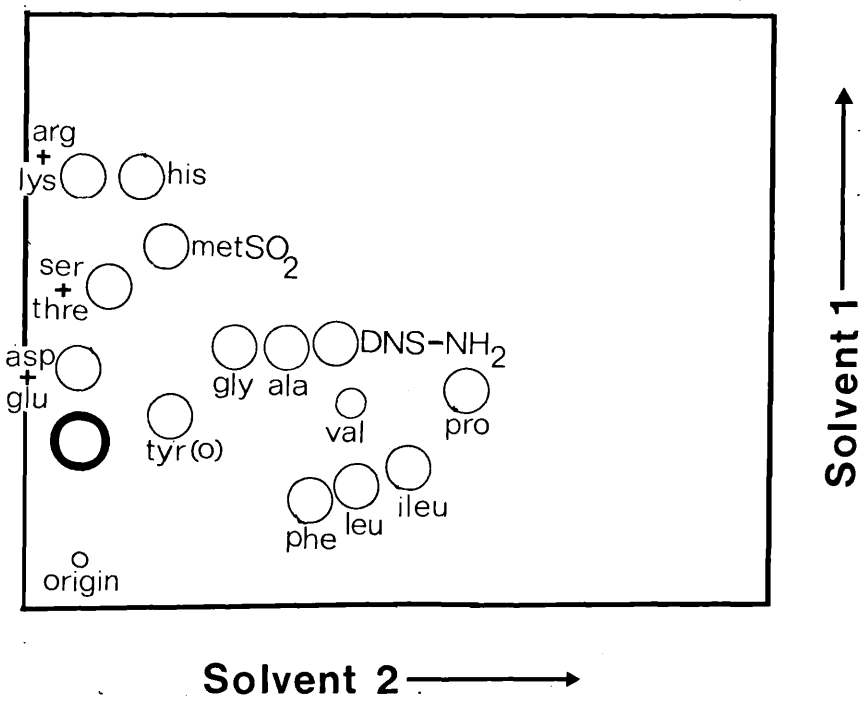

arg
+
lys
his
metSO2
ser
+
thre
asp
+
glu
gly
ala
DNS-NH2
val
pro
tyr(o)
ileu
phe
leu
origin
Solvent 1
Solvent 2

Figure 9

After development in the first solvent, the sheet was dried, turned through 90°, and run in the second solvent. After drying, the sheet was first examined under short-wave (254nm) UV light before running in the third solvent in the same direction as solvent 2.

Figure 9 shows the separation of a standard mixture of known dansylated amino acids by this system.

Electrophoresis

Samples were applied as thin streaks close to the anodic end of the paper (Whatman 3MM) as no dansyl amino acids have a negative charge. The paper was carefully wetted with pH 1.9 electrophoresis buffer and subjected to electrophoresis at 3,000 volts for 30 minutes. After thorough drying, dansyl amino acids were visualised with a short-wave UV lamp.

c) Subtractive Edman degradation

The sample removed from the aqueous layer containing the peptide, was dried and hydrolysed with 6N HCl at 105-110°C for 16 hours. Its amino acid analysis trace was compared to that of the whole peptide and thus the cleaved amino acid identified. By comparing the amino acid contents of the samples removed after each cycle of degradation, the amino acid sequence of a peptide was determined.

13. Serological techniques

a) Preparation of H antigen

H antigen was prepared as described by Edwards and Ewing (1972).

Approximately 20ml of nutrient broth was inoculated with well flagellated bacteria from the outer edge of a swarm obtained on Edward's semi-solid agar, and grown overnight at 37°C. The cells were spun down at 9,000 r.p.m., washed with saline (0.85% NaCl), resuspended in saline containing 1% formalin, and incubated at 37°C overnight. After centrifugation, the formalinised saline was removed and the cells

washed with saline. The sterility of the preparation was checked before its injection into rabbits.

b) Antiserum production

Rabbits were injected with the H antigen, prepared as described above, subcutaneously into their backs. There was an interval of 4-5 days between injections. The first dose was the smallest; 0.5ml of a suspension of about 10^6 cells/ml. This was increased to 1ml for the second dose, 2ml for the third, and 4ml for the fourth and fifth doses. The rabbits were bled from a vein in the ear before any injections were administered (control antiserum) and 6-8 days after the last injection.

c) Absorption of antisera

Antisera were absorbed according to the method of Yamaguchi and Iino (1969).

Highly motile organisms of a strain used for absorption were grown in nutrient broth overnight at 37°C. This broth was used to inoculate trays containing enriched agar medium (see "Production and Isolation of Flagella"). After 24 hours incubation at 37°C, the bacteria were harvested in 0.5% formalinised saline. The suspension was kept at room temperature overnight, then centrifuged, resuspended in sterile saline and kept at 0°C. The suspension was centrifuged before use, and the pellet mixed with the antiserum to be absorbed in the proportion of approximately 0.5gm wet weight organisms : 1ml serum. After 2 hours incubation at 37°C, the mixture was centrifuged to remove the bacteria. If this once-absorbed antiserum still agglutinated the absorbing organisms, the process was repeated until no activity with the absorbing organisms remained.

d) Titration of antisera

Antisera were titred using the slide agglutination technique.

Two-fold serial dilutions of antisera were prepared in saline. An appropriate bacterial suspension was prepared by growing highly motile organisms in nutrient broth overnight at 37°C, centrifuging, and resuspending in a small volume of saline. One drop of a dilution of antiserum was added to one drop of bacterial suspension on a microscope slide, mixed in a circular motion for about 30 seconds, and observed for agglutination. Antigen/antibody mixtures that appeared to be negative were checked under the low power objective of the microscope for weak agglutination. The titre was expressed as the reciprocal of the highest dilution showing activity.

e) Immobilisation and Immobilisation-Inhibition Technique

Serum antibodies were estimated by the immobilisation technique as described by Ada et al. (1964). Dilutions of sera were tested for their ability to immobilise a standard preparation of motile Salmonella bacteria of the appropriate antigenic type. A standard degree of partial (approximately 80%) immobilisation of the bacteria was taken as the endpoint of the titration and the reciprocal of this dilution of serum termed the titre. An inhibition technique was also used (Parish, Wistar and Ada, 1969) in which the ability of an antigen to neutralise the immobilising activity of a standard dilution of antiserum was measured. In this technique, 2-fold serial dilutions of antigen (0.25ml) were mixed with a dilution of standard antiserum (0.25ml). After the mixtures had stood at room temperature for 30 minutes, serial 2-fold dilutions of each mixture were made (final volume of each, 0.5ml) and 0.25ml of the bacterial suspension was added. These mixtures were incubated for 30 minutes at room temperature and then the dilution of antigen at which immobilisation of bacteria just occurred was read, using the "hanging drop" method, at 400-fold magnification.

f) Micro-complement Fixation and Complement Fixation Inhibition

The method used was that described by Levine (1978) and Wasserman

and Levine (1961).

The complement system of fresh guinea pig serum has the capacity to combine irreversibly with antigen-antibody complexes. If the antigen is associated with the sheep erythrocyte cell surface, such a combination may result in lysis of the erythrocyte and thus offer an excellent indicator system for complement (c) activity. Thus if complement is allowed to incubate with the antigen-antibody system under study then its combination can be estimated by the residual haemolytic activity it possesses when a known quantity of antibody-coated (sensitised) erythrocytes, is added at a later time.

Preparation of standardised red cells

Approximately 8ml sheep blood (Oxoid) preserved in Alsevers solution was centrifuged, the plasma removed, and the cells resuspended in saline (0.85% NaCl). The washing procedure was repeated at least three times until the supernatant was clear. 1ml of packed cells was then evenly suspended in 19ml of saline. This 5% cell suspension was standardised by lysing 1ml with 14ml 0.1% Na_2CO_3. A cell suspension containing 1 x 10^9 red cells/ml will have an optical density of 0.680 at 541nm in a cuvette having a pathlength of 1cm. The 5% lysed cell suspension usually had a higher reading and thus was adjusted to give a final optical density of 0.680 by appropriate dilution.

Sensitisation of sheep erythrocytes

The haemolytic rabbit antibody was obtained from Gibco, Grand Island, N. York. A stock antiserum dilution of 1:50 in saline was stored at -20°C.

For sensitisation of the cells, 10ml of the antibody, diluted 1: 1,000 were added slowly to 10ml standardised cell suspension in a 500ml Erlenmeyer flask with constant mixing. After incubation at 37°C for 15 minutes to allow maximum sensitisation, 180ml of saline were added,

and the cell suspension, which now contained 5×10^7 sensitised cells (EA) per ml, was kept at 0°C until used.

Complement

Lyophilised guinea-pig serum (Gibco, Grand Island, N. York) was firstly reconstituted with the supplied diluent. It was stored in small quantities at -20°C to minimise the loss of complement activity which may occur following repeated freezings and thawings.

Complement was titred as follows:-

To a series of 40ml centrifuge tubes in an ice-bath were added, in order, 5ml saline and 1ml complement, of various dilutions. After incubation for 16-18 hours at 2-4°C, 1ml of sensitised cells was added, and haemolysis allowed to proceed at 37°C with occasional swirling for 60 minutes. The reaction mixtures were centrifuged to sediment the unlysed cells and the absorbance of the supernatant fluid was read at 413nm.

That dilution of complement which gives 90% haemolysis is used in the complement fixation test.

Micro-complement Fixation

To a series of 40ml centrifuge tubes in an ice-bath were added, in order:

1ml diluted antiserum

3ml saline

1ml diluted complement (previously titred)

1ml antigen solution serially diluted 2-fold

Appropriate dilutions of antigen (usually highest concentration) plus complement, antibody plus complement, saline plus complement, and saline in a total volume of 6ml served as controls, and were included in every experiment. After incubation at 2-4°C for 16-18 hours, 1ml sensitised cells was added and haemolysis allowed to proceed, with

occasional swirling, in a waterbath at 37°C until the controls were visually estimated to be 80-90% haemolysed (about 30 minutes).

After immersion in an ice-bath to stop haemolytic reaction, mixtures were centrifuged for 10 minutes to sediment unlysed cells, and the optical density of the supernatant was determined at 413nm. Providing that the antigen and antibody controls do not give evidence of independent pro- or anti-complement activity, the results may be expressed as % complement fixed, where

$$\% \text{ complement fixed} = \frac{\text{Reaction } \Delta OD}{\text{Control OD}} \times 100$$

and where ΔOD equals the difference between the average optical density of the controls and the optical density of the experimental tubes.

The dilution of antiserum to be used in the above procedure was found empirically:

Undiluted rabbit serum was first heated to 60°C for 20 minutes to destroy endogenous complement activity. Complement fixation was then performed with varying dilutions of the antiserum (1/200, 1/400, 1/800 etc.) using a suitable range of antigen concentrations. The dilution of antiserum which gave a complete complement fixation curve with maximum fixation around 70% was then used for subsequent studies.

Complement Fixation-Inhibition Procedure

Investigation of inhibition by flagellin fragments of antigen-antibody interaction as measured by complement fixation was performed in the same manner as micro-complement fixation except that the antigen was kept constant and the diluent contained various quantities of inhibitor.

The constant quantity of antigen used was the amount that gave maximum complement fixation in the system described above.

To a series of 40 ml centrifuge tubes in an ice-bath were added,

in order:

1ml diluted antiserum

2ml saline

1ml diluted complement

1ml inhibitor serially diluted 2-fold

The contents were mixed well, then:

1ml antigen solution

Appropriate dilutions of inhibitor (usually highest concentration) plus complement, antibody plus complement, antigen plus complement, saline plus complement and saline served as controls and were included in every experiment.

The rest of the procedure was as described for micro-complement fixation.

Providing inhibitor, antigen and antibody controls did not give evidence of independent pro- or anti-complement activity, the inhibition was expressed as:

$$1 - \frac{\text{Complement fixed in presence of inhibitor}}{\text{Complement fixed in absence of inhibitor}} \times 100$$

14. Chemical Modification of specific amino acids in Proteins

a) Tyrosine residues

Tyrosine residues were modified by reaction with tetranitromethane (Sokolovsky et al., 1966) to give nitrotyrosine. Tetranitromethane and authentic 3-nitro-L-tyrosine were obtained from Sigma Chemical Company, St. Louis, Missouri.

Tetranitromethane was diluted 1:10 with 95% ethanol to give an 0.84M ethanolic tetranitromethane solution. 20µl of this diluted solution was added to a solution of flagellin or flagella (1mg/ml) at 20°C in 0.05M Tris-HCl buffer, pH 8.0. The reaction was allowed to proceed for one hour with occasional mixing; excess reagent was removed by dialysis against 0.05M Tris-HCl buffer, pH 8.0 at 4°C. The dialysed

material was evaporated down and hydrolysed in 0.4ml 6N HCl at 105°C for 16 hours. The tyrosine and nitro-tyrosine contents were determined by quantitative amino acid analysis.

b) Arginine residues

Arginine residues were modified by reaction with 1,2 cyclohexanedione (Patthy and Smith, 1975a,b). 1,2-cyclohexanedione (98%) was obtained from Aldrich Chemical Company Ltd., Gillingham, Dorset, England.

5.6mg of cyclohexanedione were added to a solution of flagellin or flagella (1mg/ml) in 0.2M sodium borate buffer, pH 9.0 to give a final concentration of 0.05M cyclohexanedione. The reaction was allowed to proceed at 37°C for 2 hours. Excess reagent was removed by dialysis against 1% acetic acid at 4°C. The dialysed material was evaporated down and hydrolysed in 0.4ml 6N HCl containing 20µl mercaptoacetic acid at 105°C for 16 hours. The content of modified and unmodified arginine residues was determined by quantitative amino acid analysis. Control samples were obtained for both methods by repeating the experiments in the absence of modifying reagents.

c) Lysine residues

(i) Citraconylation

Protein at 15mg/ml was treated with an approximately 10-fold excess (w/w) of citraconic anhydride (Sigma Chemical Company) over lysine residues (Dixon and Perham, 1968) while the pH was maintained at 8.0 by the addition of 3N NaOH. The reaction was complete within about 5 minutes, and the protein was desalted by dialysis against several changes of water at 4°C.

To unblock lysine residues blocked by citraconylation, the pH was lowered to 3.0 by the addition of glacial acetic acid, and the mixture incubated overnight at room temperature.

(ii) Trifluoroacetylation

The method used was that of Goldberger (1967). 100mg protein was dissolved in 25ml water and stirred continuously with a magnetic stirrer. The electrodes of a pH meter were immersed in the solution for constant monitoring of pH throughout the procedure. The pH of the solution was raised to 10.0 by addition of 1N KOH and the reaction started by the addition of 1.3ml S-ethylthioltrifluoroacetate (Sigma Chemical Company). The pH was maintained at 9.95 - 10.0 by the addition of 1N KOH. The reaction was allowed to proceed for about 1 hour at room temperature.

The trifluoroacetylated protein was precipitated from solution by adjusting the pH of the reaction mixture to 6.0 with HCl. In order to avoid overshooting the desired pH, 3ml of 0.1M sodium acetate buffer, pH 5.0 was added first. The entire reaction mixture was then added to 4 volumes of absolute ethanol at -10°C. This ensured precipitation of all the protein. The rest of the procedure was carried out at 3°C: the precipitate was collected by centrifugation, washed twice with ethanol:sodium acetate buffer (5:1, v/v) and resuspended in 25ml ethanol:sodium acetate mixture. Any last traces of unreacted reagents and side products were removed by exhaustive dialysis against 0.001M HCl, the precipitate collected by centrifugation and freezedried.

To unblock lysine residues, the trifluoroacetylated protein (10mg) was dissolved in 0.5ml 1M piperidine (grade 1, approx. 99%) at room temperature and immediately chilled in an ice-bath. After standing at 0°C for 2 hours, the reaction mixture was slowly added, with constant stirring, to a sufficient volume of 0.5M acetic acid to bring the pH to 6.0. The reagents were removed by dialysis against 0.005M acetic acid.

RESULTS

1. CYANOGEN BROMIDE CLEAVAGE OF S. SENFTENBERG g,s,t FLAGELLIN

a) Cyanogen bromide (CNBr) digestion

To obtain large fragments of the protein, S. senftenberg g,s,t flagellin was digested with cyanogen bromide. The reaction of flagellin with CNBr in dilute hydrochloric acid (0.1N) was found to be incomplete; 70% formic acid, however, was a more suitable solvent. Decreasing the concentration of formic acid to 50%, reducing the period of digestion from 18 hours to 2 hours, or treating the flagella twice with cyanogen bromide, did not appear to effect the efficiency of digestion.

(i) Polyacrylamide gel electrophoresis of CNBr digests

Polyacrylamide gel electrophoresis of CNBr digests at pH 2.7 in 8M urea revealed a series of faster running bands relative to the parent flagellin (Figure 1·1). On the basis of results obtained by Parish and Ada (1969) for S. adelaide flagellin, the five fainter bands near the top of the gel, two of which had a mobility similar to that of flagellin itself, were assumed to be degraded flagellin. The addition of mercaptoethanol during CNBr digestion, to reduce any methionine sulphoxide present to methionine, failed to eliminate these bands. The next four major bands are termed fragment A, fragment B, fragment C and fragment D in the order of their increasing mobility in the polyacrylamide gels. The number of CNBr fragments expected from a protein with three methionine residues is four. An additional band was present between fragments B and C which was a presumed 'C,D' complex.

CNBr digests of the Salmonella g.... antigenic complex flagellins, g,m; g,p and f,g showed an identical pattern of bands on pH 2.7 polyacrylamide gels in the presence of 8M urea.

(ii) Fractionation of CNBr fragments by gel filtration

The best resolution of the CNBr fragments was obtained using a

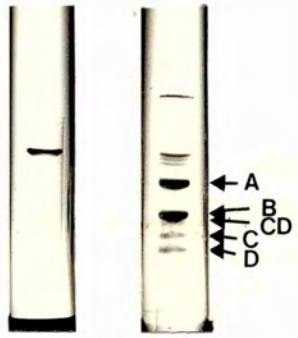
FIGURE 1·1

Polyacrylamide gel electrophoresis of S. senftenberg (SL 736) g,s,t flagellin at pH 2.7 in 8M urea.

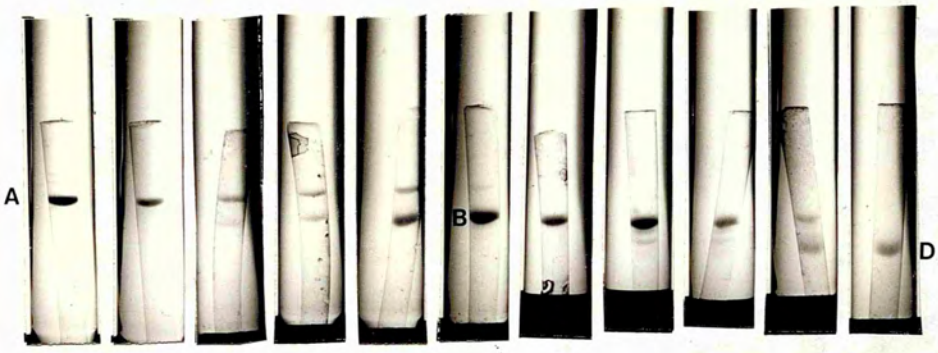
a Flagellin.

b Cyanogen bromide-treated flagellin.

c Selected gel filtration fractions of cyanogen bromide-treated flagellin.



a b



c

Sephadex G-75 (40-120μ) column, dissolving the CNBr digest in 70% formic acid and eluting with 5% formic acid. The use of 8M urea as a solvent was not found suitable on a range of Sephadex columns. Selected fractions were monitored by polyacrylamide gel electrophoresis at pH 2.7 in 8M urea. Comparison with the whole CNBr digest, run on a corresponding gel, enabled the CNBr fragments to be located (Figure 1·1). Appropriate fractions were pooled, freezedried and subjected to polyacrylamide gel electrophoresis at pH 2.7 in 8M urea to determine the homogeneity of the fragments.

An alternative method of monitoring fractions, involving the measurement of turbidity of the fractions at 420nm after the addition of 6% trichloroacetic acid, was not found suitable and, in addition, required a larger amount of sample than polyacrylamide gel electrophoresis. It was, therefore, not used routinely.

Sephadex G-75 (40-120μ) has an effective fractionating range of approximately 40,000 down to 5,000 and so, although the larger CNBr fragments A and B were well resolved on the column, the smaller fragment C and the 'C,D' complex were never obtained free from fragments B and D. These unresolved mixtures were pooled and re-run on either a Sephadex G-75 (40-120μ) or Sephadex G-50 (Fine) column. The latter gel has an effective fractionating range of about 15,000 down to 2,000, and should, therefore, give a better resolution of the smaller CNBr fragments. However, it was not possible to obtain pure CNBr C fragment or 'C,D' complex in any quantity by these techniques.

When the amount of CNBr digest was increased to 400mg and applied to a larger Sephadex G-75 (40-120μ) column, that is, 100cm x 5cm, instead of 100cm x 2.5cm, unsatisfactory fractionation was obtained on several occasions. It was possible, however, to apply up to 200mg CNBr digest to the smaller column if the sample was dissolved in 98-100% formic acid (thus preventing gelling), and if its addition was preceded

and followed by 70% formic acid.

(iii) Chloramine-T Oxidation

When polymerised flagellin was exposed to relatively high concentrations of the oxidant chloramine-T and then treated with CNBr, a simpler pattern of breakdown products was observed on polyacrylamide gel electrophoresis at pH 2.7 in 8M urea (Figure 1·2). Of the two strongly staining bands at the top of the gel, one corresponds to that of the parent flagellin, and the other probably represents an 'A,B' complex. The other main band corresponds in position to the 'C,D' complex. Lighter bands of fragments A and B are also present.

Comparison of polyacrylamide gel electrophoresis of chloramine-T treated and native flagellin (Figure 1·2) indicated that chloramine-T did not degrade the protein to any extent.

Although the oxidation incubation time was varied, it was not possible to obtain the 'C,D' complex without the presence of CNBr A and CNBr B fragments after CNBr digestion. Gel filtration on a Sephadex G-75 (40-120μ) column resolved CNBr A, but the 'C,D' complex remained contaminated with a small amount of CNBr B fragment.

Omission of the acid-dissociation step before oxidation did not affect the results except when native flagella were used; prior acid dissociation was then necessary for oxidation to occur.

Presumably, chloramine-T oxidises the more exposed methionine residues to methionine sulphoxide, which is not susceptible to CNBr cleavage. Thus the methionine residue linking CNBr C and CNBr D must be very accessible to chloramine-T, whereas the methionine residue linking 'C,D' complex to the rest of the flagellin molecule is probably masked and therefore less readily oxidised. The presence of small amounts of CNBr A and CNBr B after CNBr cleavage of chloramine-T treated flagellin suggest that the methionine residue linking CNBr A and CNBr B must be at least partially inaccessible to chloramine-T.

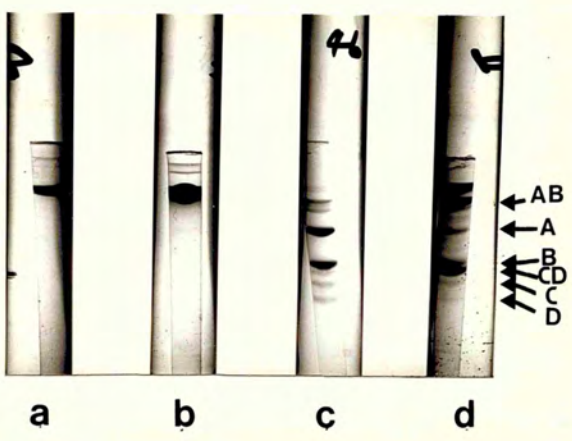


FIGURE 1·2

Polyacrylamide gel electrophoresis of S. senftenberg (SL 736) g,s,t flagellin at pH 2.7 in 8M urea.

a Flagellin.

b Chloramine-T oxidised flagellin.

c Cyanogen bromide-treated flagellin.

d Cyanogen bromide-treated, chloramine-T oxidised flagellin.

b) Amino acid composition of flagellin and its CNBr fragments

Flagellin

(i) Reproducibility and error

Table 1·1 shows the amino acid analysis of two hydrolysis samples of g,s,t flagellin; analysis of hydrolysis sample (1) has been duplicated. These results indicate the reproducibility of the technique as shown by the two hydrolysis samples which gave closely similar analyses. Duplicate runs of the same hydrolysis sample indicate the small amount of error (difference of one, or at most, two residues for each amino acid) involved.

To correct for the degradation of threonine and serine, and the incomplete hydrolysis of bands adjacent to valine and isoleucine residues, correction factors of 3% (threonine), 6% (serine), and 5% (valine and isoleucine) were introduced into the calculations at the nmole level for all amino acid analyses carried out. Addition of phenol, thioglycollic acid or mercaptoethanol during hydrolysis did not appear to prevent oxidation of tyrosine or methionine residues and was therefore omitted from the procedure.

In every case, unless otherwise stated, the values given represent the number of residues calculated for a molecular weight of 40,000 from analyses of 24 hour hydrolysates.

The presence of tryptophan was not investigated since it has always been reported absent in bacterial flagellins.

(ii) Amino acid compositions of Salmonella g.... antigenic flagellins.

The amino acid compositions of ten g.... antigenic flagellins are presented in Table 1·2. All the flagellins had a high proportion of aspartic and glutamic acids (or amides) and alanine, and little tyrosine, phenylalanine, methionine and proline. Cysteic acid or cysteine was never detected; nor was histidine except in the case of

AMINO ACID	HYDROLYSIS 1: ANALYSIS 1	HYDROLYSIS 1: ANALYSIS 2	HYDROLYSIS 2
Asp	68	67	68
Thre	36	35	34
Ser	35	36	34
Glu	35	37	37
Pro	4	4	3
Gly	31	29	31
Ala	46	47	48
Val	27	29	28
Met	3	3	3
Ileu	23	24	22
Leu	26	26	27
Tyr	7	7	7
Phe	11	11	10
His	0	0	0
Lys	26	27	26
Arg	11	10	12
TOTAL	389	392	390

TABLE 1·1 Amino acid analysis of S. senftenberg (SL 736) g,s,t flagellin.

AMINO ACID	g,s,t (SL 736)	g,m (SL 588)	g,p (SL 166)	f,g (SL 175)	g,t (NCTC 5724)	m,t (NCTC 5743)	g,m,s (NCTC 5747)	g,q (NCTC 10480)	g,p,u (NCTC 5767)	g,m,t (NCTC 6018)
Asp	68	62	61	58	64	60	63	55	60	59
Thre	34	33	32	35	39	34	32	38	34	37
Ser	34	30	32	35	40	38	30	36	32	39
Glu	37	35	34	35	36	35	39	31	38	35
Pro	3	4	4	4	3	5	3	7	3	4
Gly	31	34	32	33	29	27	32	31	31	27
Ala	48	47	50	49	42	43	45	55	44	44
Val	28	29	32	27	27	30	31	33	31	31
Met	3	2	3	3	2	3	2	3	2	2
Ileu	22	25	25	23	23	21	23	24	24	24
Leu	27	28	28	30	29	27	29	32	29	27
Tyr	7	7	6	7	7	8	6	12	7	6
Phe	10	10	10	10	8	12	9	8	8	8
His	0	0	0	0	0	0	0	1*	0	0
Lys	15	15	15	26	27	27	26	22	27	29
NML	11	14	11	0	E^+	E^+	E^+	E^-	E^+	E^+
Arg	12	12	12	12	11	11	10	13	11	11
Total	390	387	387	385 387	387	381	382	401	380	383

TABLE 1·2 Amino acid compositions of Salmonella g.... antigenic flagellins

E^+ Presence of NML
E^- Absence of NML
} as indicated by pH 3.5 paper electrophoresis of hydrolysate.

* Unconfirmed

g,q flagellin. The latter flagellin, in addition to the possible presence of histidine, possessed certain features that were more similar to other Salmonella antigenic types, such as 1,2; a; b; r; e,h; and e,n,x than to the g.... antigenic flagellins. Lower aspartic acid and lysine levels, higher proline and alanine levels and a reversed phenylalanine/tyrosine ratio as compared to the other g.... antigenic flagellins were noted.

In some cases, a low value for methionine (2 residues/molecule) was obtained. The presence of four fragments after CNBr digestion of g,s,t; g,m; g,p and f,g flagellins suggests that their methionine content should be 3 residues/molecule.

(iii) Presence of ε-N-methyllysine in Salmonella g.... antigenic flagellins.

The presence of ε-N-methyllysine (NML) in g.... antigenic flagellins was determined either quantitatively (automatic amino acid analysis) or qualitatively (pH 3.5 paper electrophoresis) (Table 1·2).

Amino acid analysis revealed a NML/lysine ratio of 0.73 for g,s,t (Figure 1·3) and g,p flagellins, and 0.93 for g,m flagellin. NML was not detected in f,g flagellin.

Hydrolysates of the other 6 g.... antigenic flagellins were subjected to paper electrophoresis at pH 3.5 in the presence of suitable markers. NML was detected in all the flagellins except that of the g,q antigenic type. This method is sensitive to only 1 NML residue/molecule flagellin, as shown in Figure 1·4.

In the flagellins where no NML residues were detected, for example, f,g flagellin, there was an increased lysine content as compared to those flagellins in which NML was present (g,s,t; g,m and g,p flagellins).

CNBr Fragments

Amino acid analyses were performed on CNBr fragments A, B and D

FIGURE 1·3

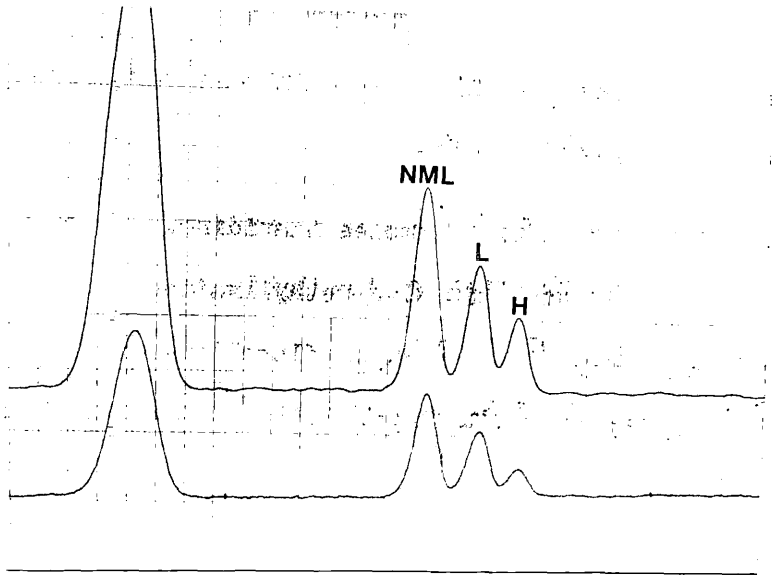
Separation of histidine (H), lysine (L) and ∈-N-methyllysine (NML) by automatic amino acid analysis

a Standard mixture of 25 nmoles histidine, 25 nmoles lysine and 50 nmoles of authentic ∈-N-methyllysine.

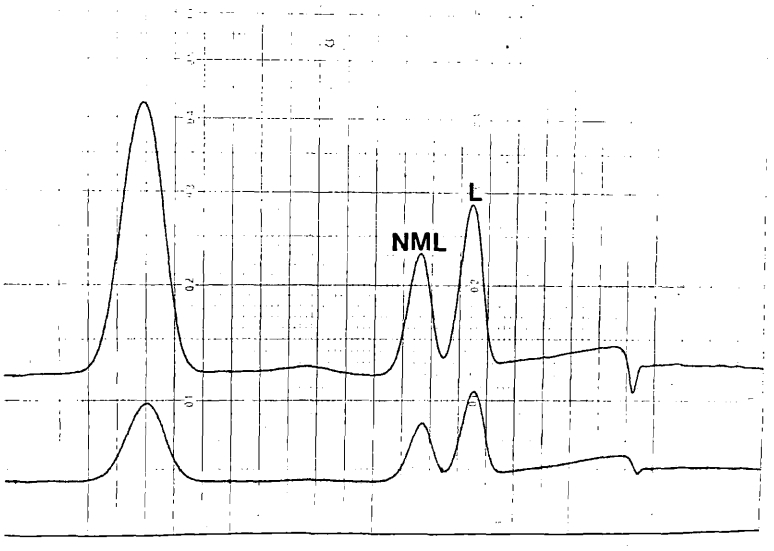
b *S. senftenberg* (SL 736) *g,s,t* flagellin.

c CNBr A fragment of *S. senftenberg* (SL 736) *g,s,t* flagellin.

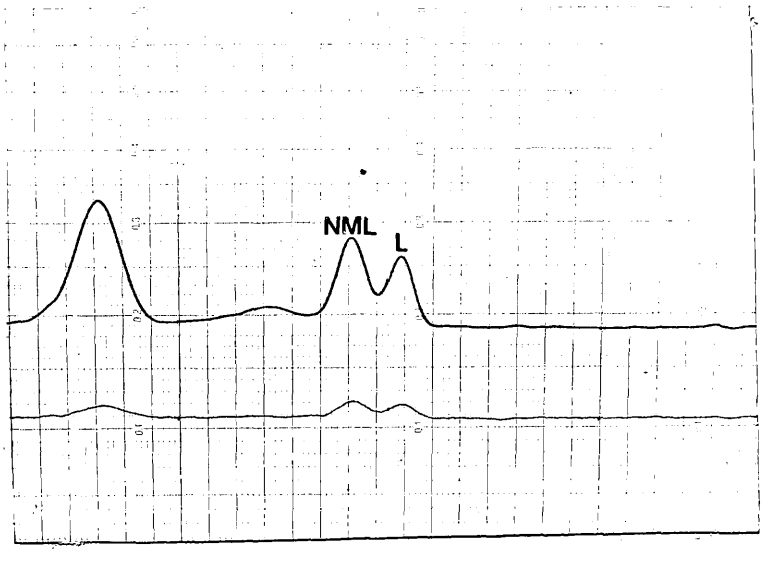
a



b



c



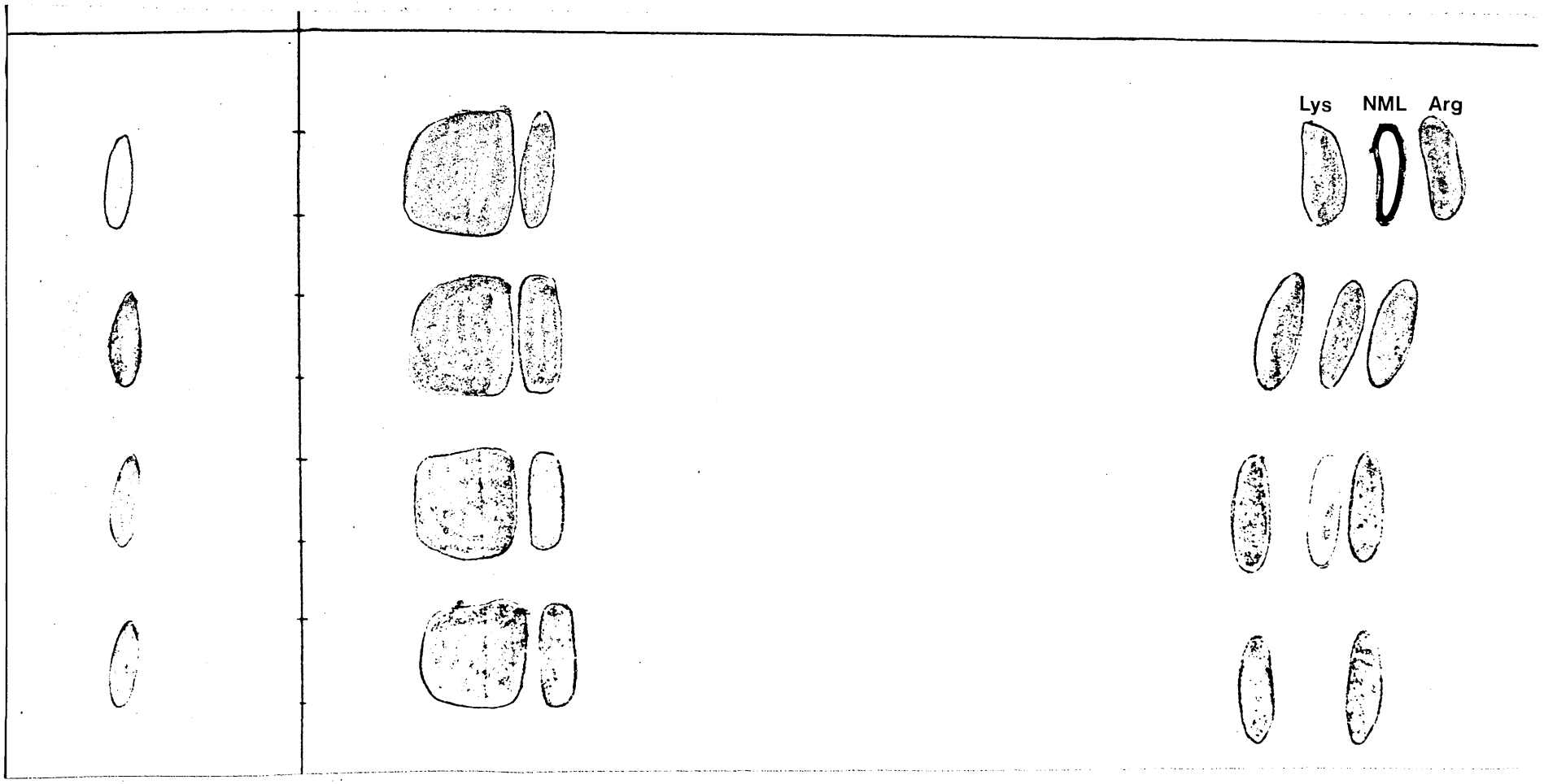


FIGURE 1·4 Paper electrophoresis, at pH 3.5, of hydrolysates containing from top to bottom: 6; 3; 1 and 0 NML residues/molecule flagellin.

and on the 'C,D' complex (Table 1·3). The sum of the analysis of fragments A, B and the 'C,D' complex agrees reasonably well with the composition of the complete flagellin molecule. An analysis for fragment C was obtained by subtraction.

Fragment A contained 4 of the 7 tyrosine residues, and 9 of the 11 NML residues of the molecule (Figure 1.3), but was low in glutamic acid, isoleucine and leucine, and contained only 1 of the 12 arginine residues. Its molecular weight was estimated to be 19,000, based on the arginine content.

Fragment B contained no tyrosine or proline, but possessed 6 of the 12 arginine residues and 16 of the 37 glutamic acid residues of the molecule.

The serine, isoleucine, leucine and phenylalanine contents of fragments A and B were very similar.

0.3 residue of methionine/molecule was detected in the 'C,D' complex, which was presumably a residue of methionine sulphoxide during CNBr digestion, which was partially reconverted into methionine by acid hydrolysis.

There were 2 NML residues unaccounted for; NML residues were not determined in CNBr B and D, and the 'C,D' complex.

Methionine is converted into homoserine lactone by treatment with CNBr, thus there should be one residue of the latter/molecule in each CNBr fragment except the C-terminal fragment. Homoserine lactone was not, however, easily resolved from serine by the amino acid analysis system used.

c) N-terminal analysis

Dansylation of flagellar protein of 9 Salmonella g.... antigenic strains: g,s,t; g,m; g,p; f,g; g,m,t; m,t; g,m,s; g,t and g,p,u followed by the hydrolysis and separation of the hydrolysis products by pH 1.9 electrophoresis, indicated that alanine was the N-terminal amino

AMINO ACID	FRAGMENT A	FRAGMENT B	'C,D' COMPLEX	TOTAL A+B+'C,D'	FLAGELLIN	FRAGMENT D	FRAGMENT C (C,D - D)
Asp	34	20	14	68	68	7	7
Thre	20	8	7	35	34	4	3
Ser	10	12	12	34	34	5	7
Glu	11	16	8	35	37	3	5
Pro	2	0	1	3	3	1	0
Gly	18	6	7	31	31	2	5
Ala	25	11	13	49	48	7	6
Val	14	6	7	27	28	3	4
Met	0	0	0.3	0.3	3	0	0
Ileu	7	8	6	21	22	3	3
Leu	9	11	8	28	27	3	5
Tyr	4	0	2	6	7	1	1
Phe	4	2	3	9	10	1	2
His	0	0	0	0	0	0	0
Lys	7	5	6	27	15	2	4
NML	9	ND	ND	-	11	ND	-
Arg	1	6	4	11	12	2	1
TOTAL	175	111	97	385	390	44	53

TABLE 1·3 Amino acid analysis of *S. senftenberg* (SL 736) g,s,t flagellin and of fragments obtained from flagellin by cyanogen bromide digestion.

Values are given as residues/molecule assuming the molecular weight of flagellin to be 40,000, that of fragment A to be 18,000, that of fragment B to be 12,000, that of the 'C,D' complex to be 10,000 and that of fragment D to be 4,500.

The molecular weight of fragment C was calculated, by subtraction, to be 5,500.

Each value is the average of two or three analyses of samples hydrolysed for 24 hours.

ND - Not determined.

acid for all the flagellins examined.

However, although comparatively large amounts of flagellar protein (about 1mg) were used, the band representing alanine was not strongly fluorescent, in contrast to bands representing dansyl-o-tyrosine and dansyl- ϵ -lysine which were easily distinguished. Because of these additional fluorescent products, the N-terminal amino acid was not easily identified by the thin-layer chromatography system of Woods and Wang (1967). However, the separation of a standard mixture of amino acids by this technique was readily achieved.

Alanine also appeared to be the N-terminal amino acid of both CNBr A and CNBr B of g,s,t, flagellin, and phenylalanine, the N-terminal amino acid of CNBr D. According to Parish and Ada (1969), CNBr B is the N-terminal peptide of S. adelaide f,g flagellin as, in addition to other experimental evidence, its N-terminal amino acid, and that of the flagellin, is alanine. However, in contrast to the results obtained for g,s,t CNBr A, the N-terminal amino acid of CNBr A of S. adelaide f,g flagellin was determined as lysine (Davidson, 1971). This difference could be the result of contamination of g,s,t CNBr A by partially degraded flagellin. On the other hand, it could represent an amino acid difference between the flagellins of the two antigenic types.

d) C-terminal analysis

Four Salmonella flagellins: g,s,t; g,m; g,p and g,m,t were digested with carboxypeptidase-B and the resulting supernatants subjected to quantitative amino acid analysis. Peaks corresponding to arginine, leucine, serine and, after longer periods of digestion, lysine were obtained. Arginine and leucine were released in the proportions of about 1:2; arginine was the first amino acid released.

For example: g,m flagellin

Amino acid analysis of supernatant after 30 minutes of carboxypeptidase-B digestion:

	nmoles	Ratio
Arginine	.101	1
Leucine	.167	1.7
Serine	.094	0.9
Lysine	.056	0.6

Thus the C-terminal sequence is probably:

..... Lys - Ser - Leu - Leu - ArgCOOH

CNBr fragments A, B and D of g,s,t flagellin were digested with a combination of carboxypeptidase A and carboxypeptidase B. Amino acid analysis of the supernatants of CNBr B and D revealed the presence of leucine and arginine; the fragments were obviously contaminated with flagellin or the C-terminal peptide which is, according to Parish and Ada (1969) and Davidson (1971), CNBr C in S. adelaide flagellin.

Methionine is converted into homoserine lactone by treatment with CNBr and thus homoserine should be the first amino acid released from each CNBr fragment except for the C-terminal peptide. However, only threonine was released from CNBr A after 30 minutes digestion. Threonine was also the second amino acid from the C-terminal end of the CNBr A fragment of S. adelaide flagellin (Davidson, 1971).

e) Polyacrylamide gel electrophoresis

(i) Determination of homogeneity

A range of Salmonella g.... antigenic flagellins were subjected to polyacrylamide gel electrophoresis at pH 2.7 in 8M urea to determine their homogeneity (Figure 1·5). In each case, a single band was obtained.

(ii) Molecular weight estimations of Salmonella g.... antigenic flagellins and their CNBr fragments

Molecular weight estimations were made using SDS polyacrylamide gel electrophoresis at pH 8.8.

Salmonella g.... antigenic flagellins ran as single bands on 12.5% gels; CNBr digests of g,s,t; g,m and g,p flagellins formed several bands

FIGURE 1·5

pH 2.7 polyacrylamide gel electrophoresis of Salmonella g.... antigenic flagellins in 8M urea.


gst
gt
gm
fg
gms
gmt
gpu
gq
gp

on both 12.5, and 15% gels (Figure 1·6).

Proteins of known molecular weight were included in each experiment, and their mobilities (distance of protein migration/ distance of dye migration) calculated. A standard curve of mobility vs. the logarithm of the molecular weight of these proteins (Figure 1·7) enabled the molecular weights of the flagellins and their CNBr fragments to be estimated from their mobilities.

The values obtained for the molecular weight of the flagellins were:

g,s,t	55,935	± 2.9%	2 runs
g,m	57,410	± 0.2%	"
g,p	57,410	± 0.2%	"
g,q	54,230	± 3.2%	"
g,t	54,015	± 0.6%	"
g,p,u	55,620	± 3.4%	"

Molecular weight values for the 3 slowest moving bands of the flagellin CNBr digests were obtained from both 12.5 and 15% gels. These bands correspond to degraded flagellin, CNBr A and CNBr B in order of their increasing mobility in the gels. The smaller CNBr fragments ran as a single diffuse band at the bottom of the gel, and thus their molecular weights could not be determined.

	Slowest moving band	CNBr A	CNBr B
g,s,t	41,215 ± 1%	26,795 ± 5%	11,655 ± 8%
g,m	41,120 ± 1%	27,080 ± 3%	11,970 ± 5%
g,p	42,415 ± 0.6%	27,550 ± 2%	12,035 ± 5%

The values were the average of 2 runs.

f) Enzymic digestion of g,s,t flagellin and its CNBr fragments

(i) Peptide maps

Trypsin was the most specific, and therefore the most frequently used enzyme for digestion of g,s,t flagellin and its CNBr fragments. Other enzymes such as chymotrypsin, thermolysin and pepsin, which gave a more complete digestion of the protein, were also employed. When

FIGURE 1·6

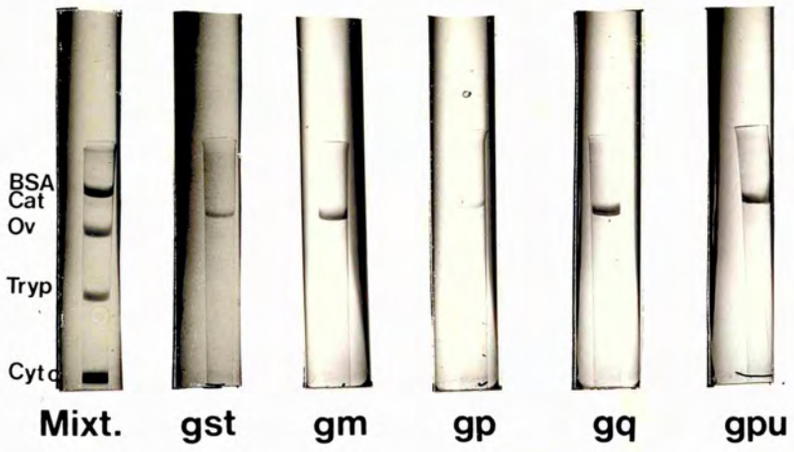
SDS polyacrylamide gel electrophoresis at pH 8.8 of Salmonella g.... antigenic flagellins and their CNBr digests.

a) Flagellins - 12.5% gels.

b) CNBr digests - 15% gels.

Marker protein mixtures were included in each run.

a


BSA
Cat
Ov
Tryp
Cyt c
Mixt.
gst
gm
gp
gq
gpu

b

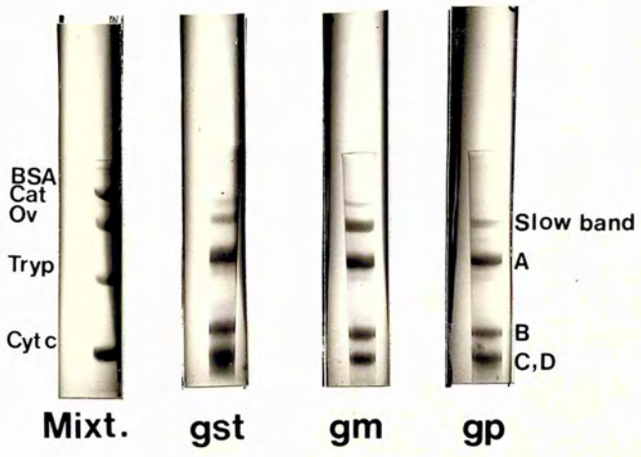

BSA
Cat
Ov
Tryp
Cyt c
Slow band
A
B
C,D
Mixt.
gst
gm
gp

FIGURE 1·7

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

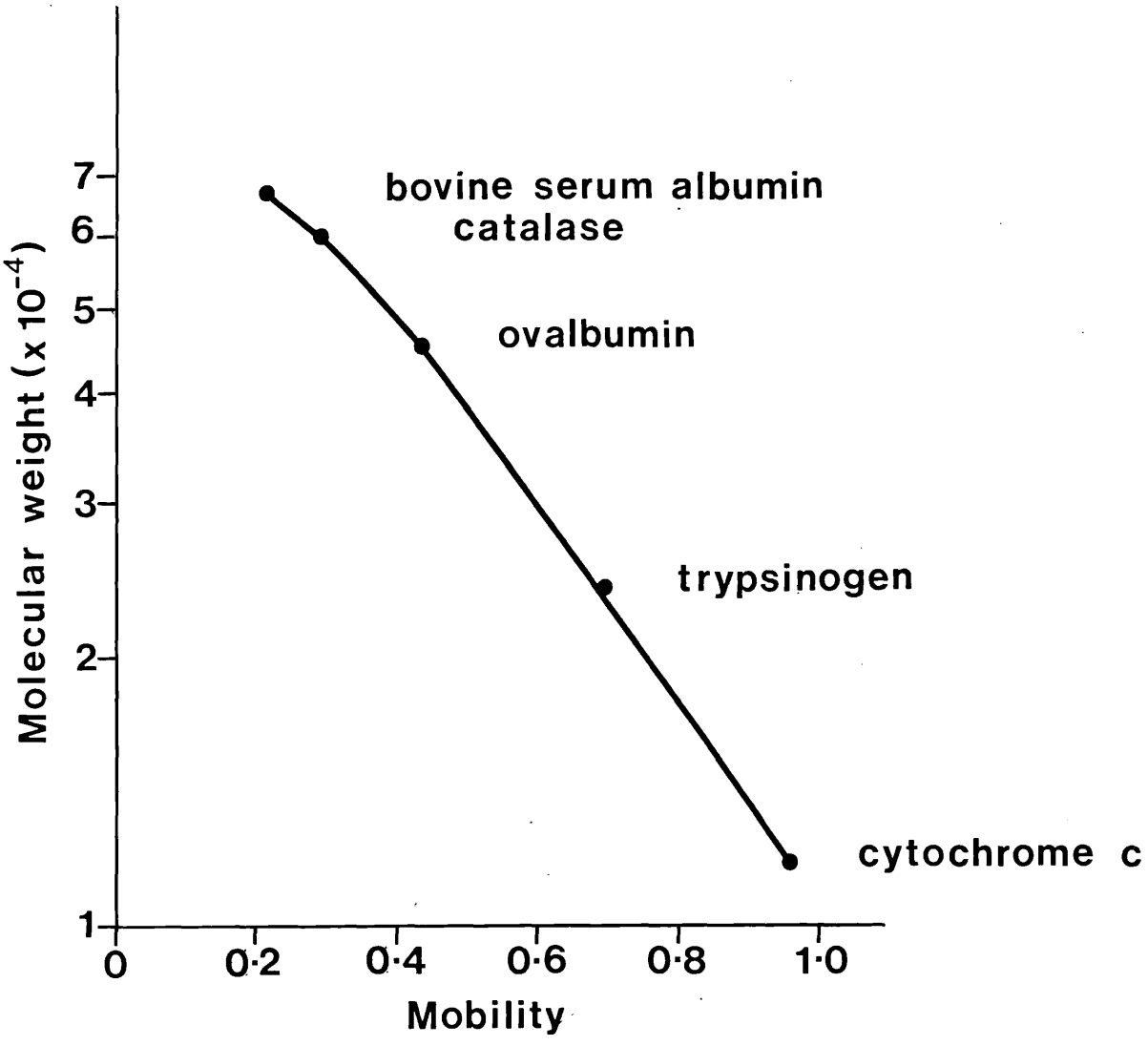

Molecular weight (x 10^{-4})
7
6
5
4
3
2
1
0
0·2
0·4
0·6
0·8
1·0
Mobility
bovine serum albumin
catalase
ovalbumin
trypsinogen
cytochrome c

Figure 1·7

digests were spotted on chromatography paper and subjected to descending chromatography followed by electrophoresis at pH 1.9 or 6.5, very reproducible peptide maps were obtained (Figures 1·8, 1·9, 1·10, 1·11 and 1·12). However, slight variations in the position of peptides and in their intensities were observed between repeat runs.

(ii) Peptides containing specific amino acids

Tryptic peptides were further characterised by tests specific for certain amino acids (Figure 1·13). The number of tyrosine- and arginine-containing peptides equalled the number of residues obtained by amino acid analysis in the whole flagellin (Table 1·4). This is to be expected if the similar residues are not clustered together but are well distributed along the polypeptide chain. However, in the CNBr fragments A and D of flagellin, the number of tyrosine- and arginine-containing peptides tended to exceed that of the corresponding residues. This could be explained by a rather low estimate of the residue number by amino acid analysis, or, perhaps more likely, some degradation of the tryptic peptides. The two arginine-containing peptides detected in CNBr A were only weakly stained and their presence, therefore, somewhat doubtful. Weak staining tyrosine- and arginine-containing peptides were also observed in the other CNBr fragments; their inclusion may explain the rather high estimates of the peptide numbers.

The number of arginine-containing and tyrosine-containing peptides in 'C,D' complex, however, were less than the corresponding number of residues. It is of interest that when trypsin digests of flagellin or its CNBr fragments are mapped, ninhydrin-positive material is invariably observed at the origin. If it is assumed that this material represents an undigested "core", then it is possible that the amino acids unaccounted for might be located in such a "core".

The total number of tryptic peptides, and the summed numbers of lysine and arginine residues as determined by amino acid analysis are

FIGURE 1.8

Enzymic peptide maps of S. senftenberg (SL 736) g,s,t flagellin:

a Trypsin

b Chymotrypsin

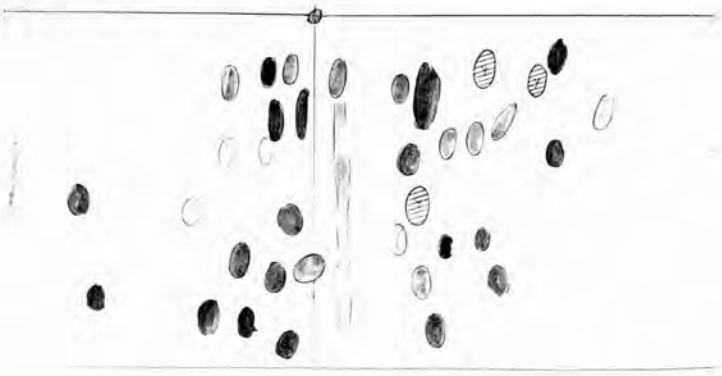
c Thermolysin

d Pepsin

 - peptide with N-terminal glycine or amide residue.

N.B.— the shading of spots on the peptide maps presented in this thesis is simulated. This was necessary due to deterioration of the ninhydrin stain used to locate peptides on the peptide maps.

a



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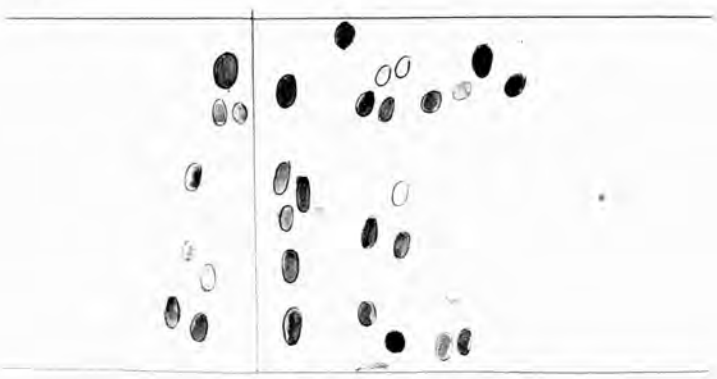

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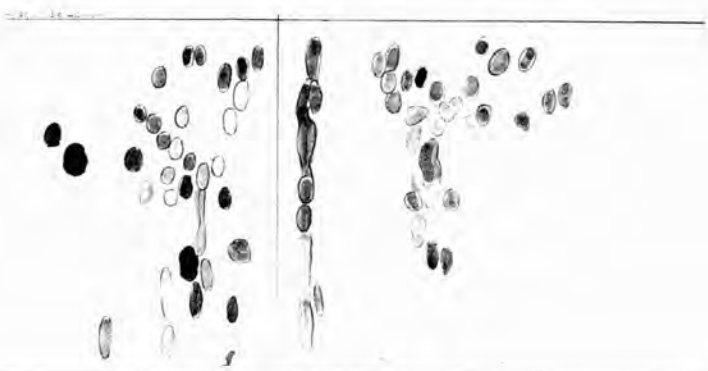

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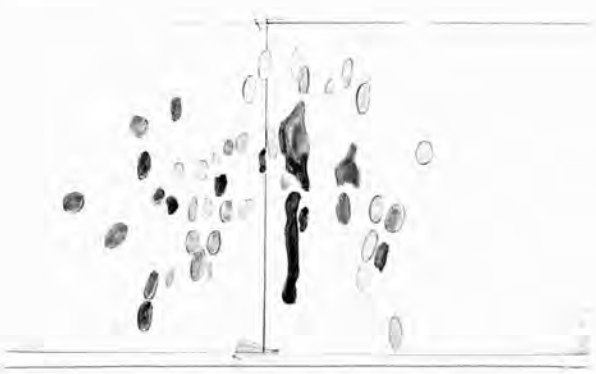


FIGURE 1·9

Enzymic peptide maps of CNBr A fragment of S. senftenberg (SL 736) g,s,t flagellin:

a Trypsin

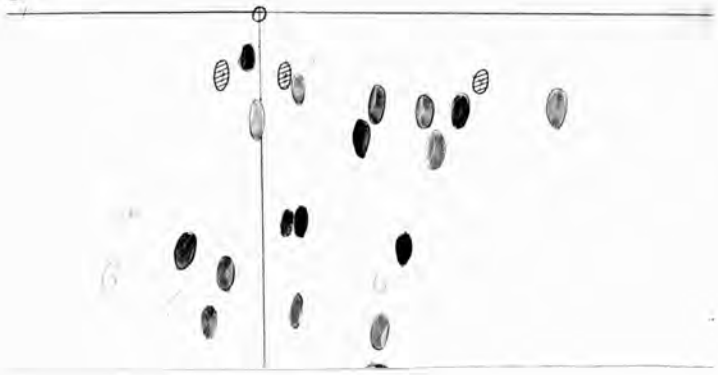
b Chymotrypsin

c Thermolysin

d Pepsin

 - peptide with N-terminal glycine or amide residue.

a



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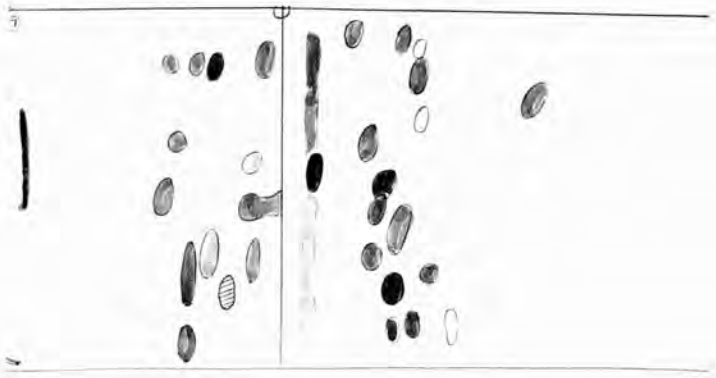

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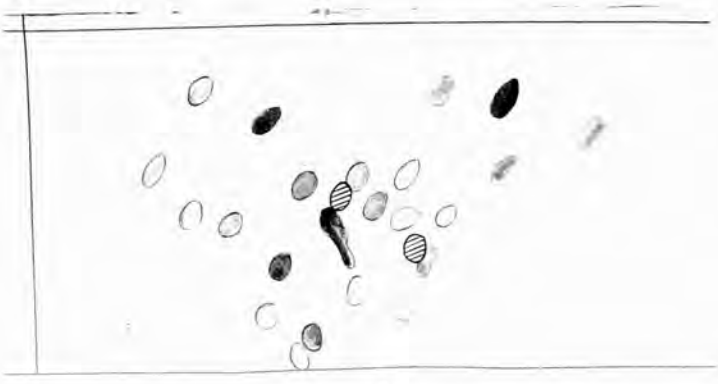

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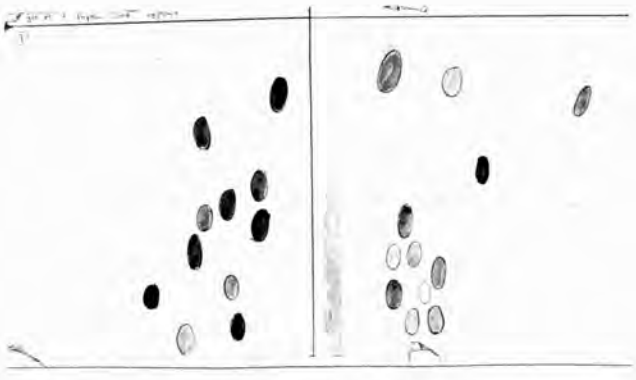


FIGURE 1.10

Enzymic peptide maps of CNBr B fragment of S. senftenberg (SL 736) g,s,t, flagellin:

a Trypsin

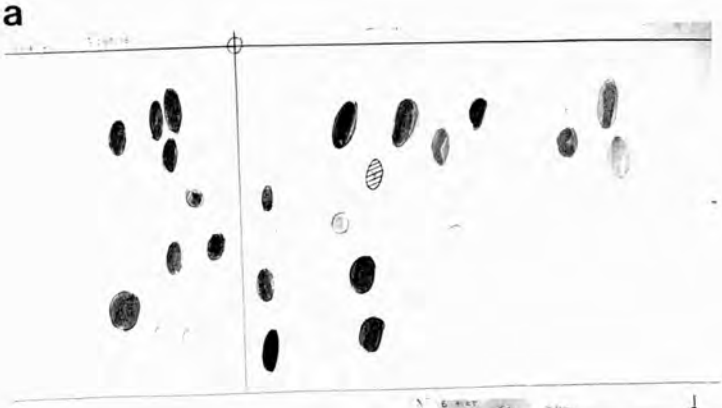
b Chymotrypsin

c Thermolysin

d Pepsin

 - peptide with N-terminal glycine or amide residue.

a



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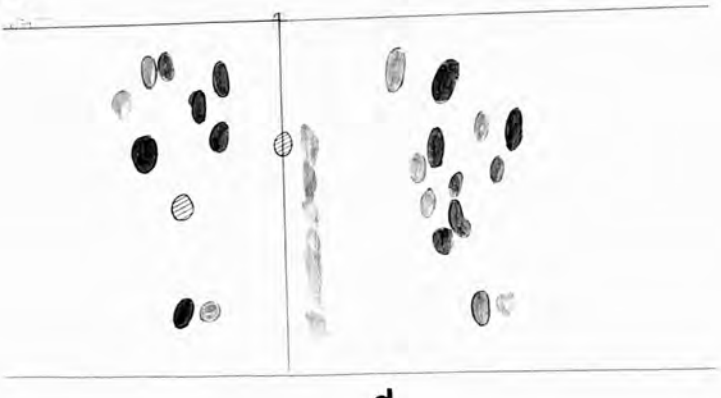
c



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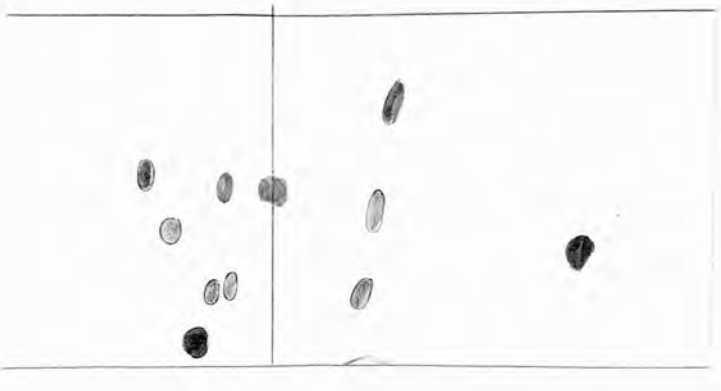


FIGURE 1·11

Enzymic peptide maps of CNBr C,D fragment of S. senftenberg (SL 736) g,s,t, flagellin:

a Trypsin

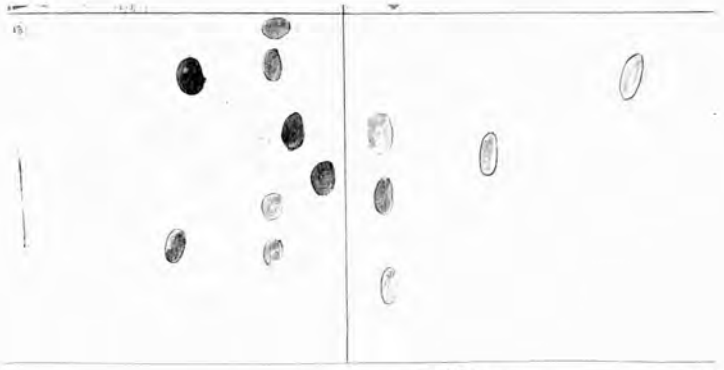
b Chymotrypsin

c Thermolysin

d Pepsin

 - peptide with N-terminal glycine or amide residue.

a



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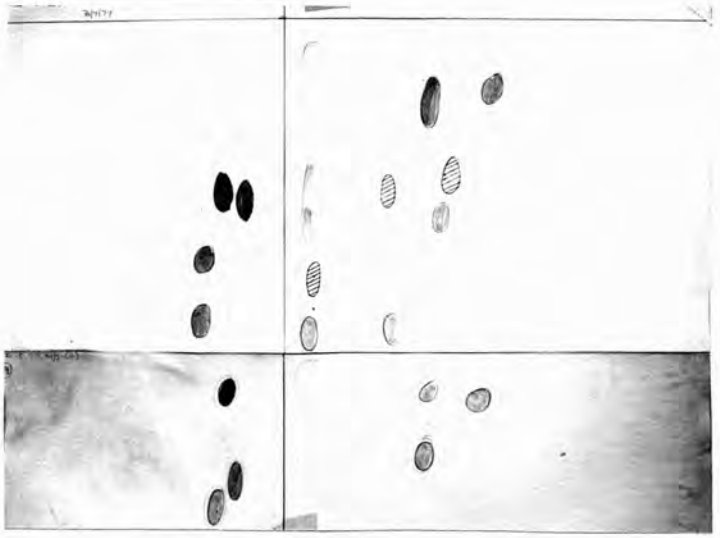

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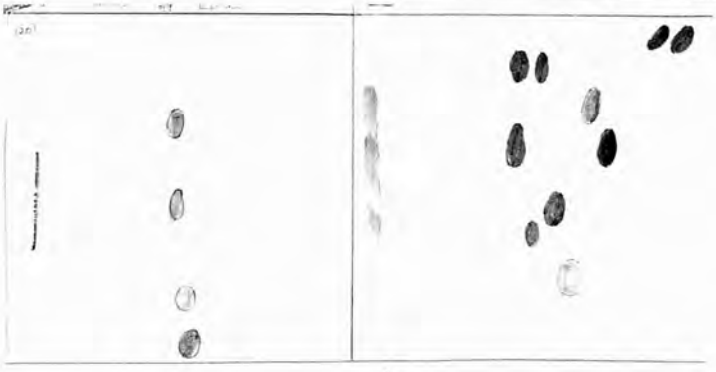

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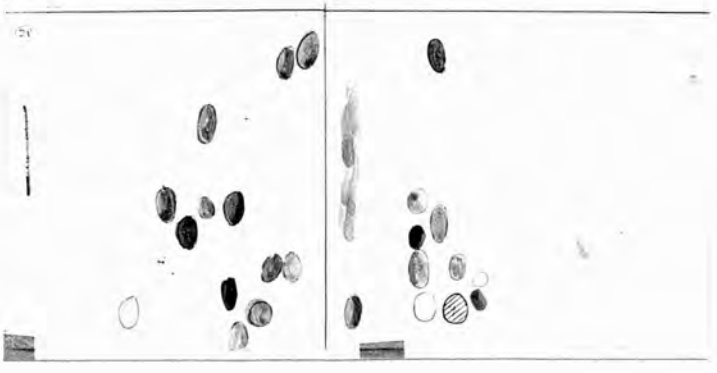


FIGURE 1·12

Enzymic peptide maps of CNBr D fragment of *S. senftenberg* (SL 736) *g,s,t* flagellin:

a Trypsin

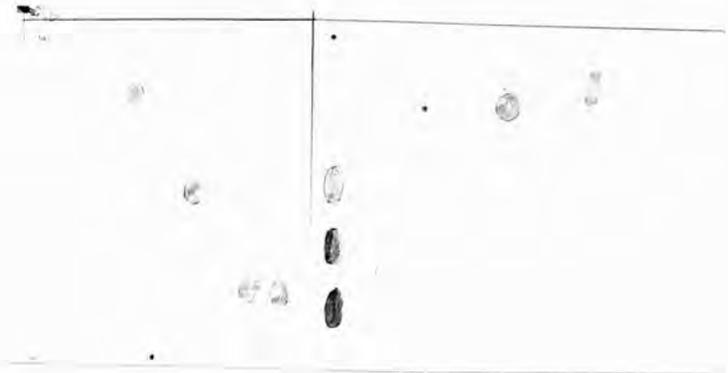
b Chymotrypsin

c Thermolysin

d Pepsin

 - peptide with N-terminal glycine or amide residues.

a



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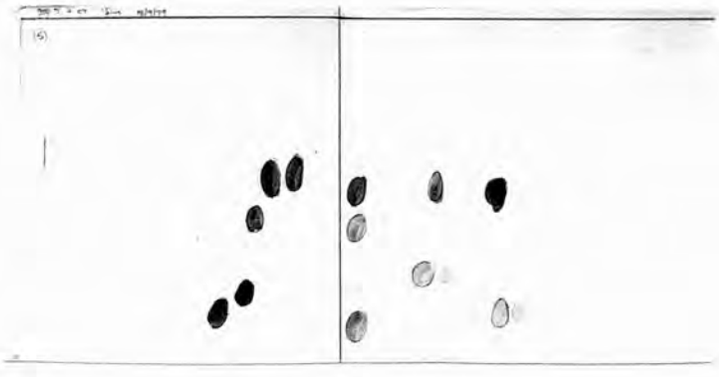

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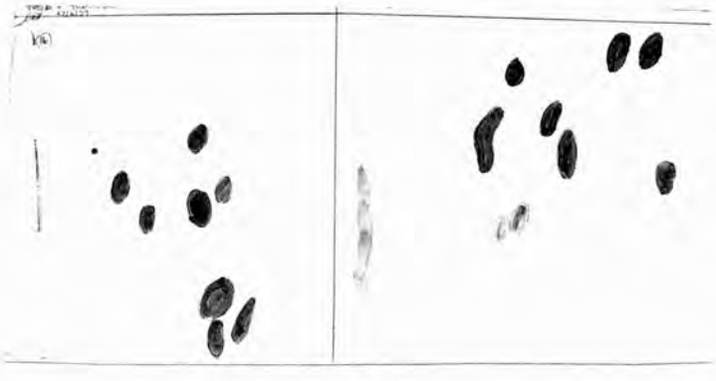

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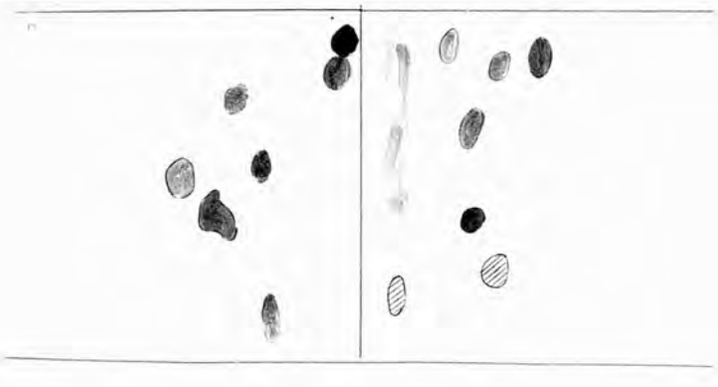


FIGURE 1·13

Tryptic peptide map of *S. senftenberg* (SL 736) *g,s,t* flagellin.


- peptides containing arginine


- peptides containing tyrosine

A, B, CD or D - CNBr fragment in which peptide is located

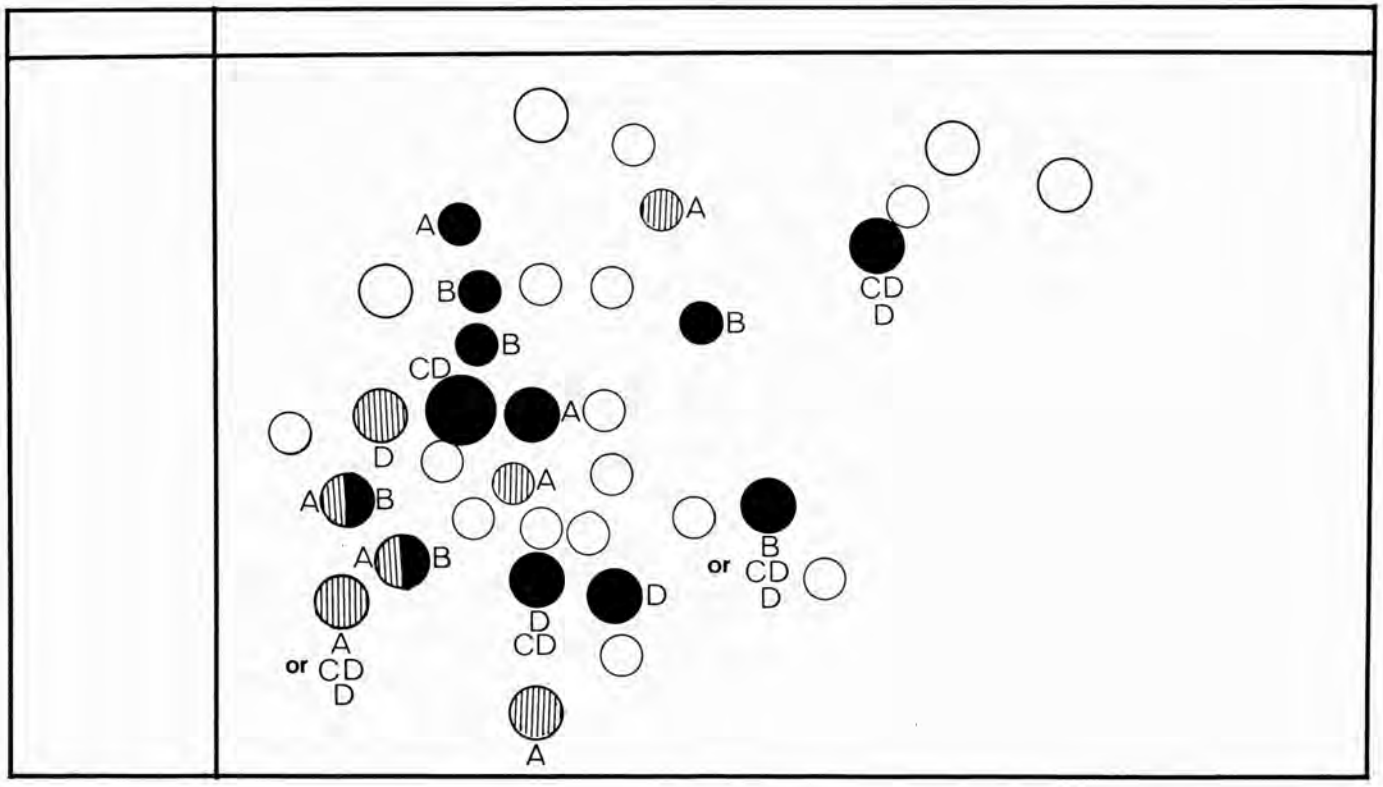


Figure 1·13

also presented in Table 1.4. If trypsin cleaves 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 the case of the whole flagellin, the actual number of peptides was slightly less than the theoretical number, whereas in the CNBr fragments, it was slightly more. In the former instance, the "missing" peptides may be located in the "core" material; the latter results may be due to breakdown of tryptic peptides.

When flagellin was digested with chymotrypsin, thermolysin or pepsin, and peptide maps prepared, the number of peptides observed was very similar to that obtained by tryptic digestion. As these enzymes are less specific than trypsin, a larger number of peptides would be expected. The tryptic peptide maps were electrophoresed at pH 1.9; at this pH all the peptides move towards the cathode. The other enzymic maps, on the other hand, were electrophoresed at pH 6.5; at this pH, the neutral peptides do not move and are, therefore, not resolved. The neutral peptides, therefore, probably represent the additional peptides expected when a less specific enzyme than trypsin is used.

g) Fractionation and sequence of peptides of g,s,t flagellin

(i) Fractionation of tryptic peptides

Despite the use of several different buffer gradients, as described in the Methods section, satisfactory resolution of the tryptic peptides of g,s,t flagellin, or of its CNBr fragment B, by ion-exchange chromatography was not obtained.

It was therefore decided to isolate CNBr B tryptic peptides by eluting them from peptide maps. Although most peptides were obtained in a reasonably pure state by this method, the yields were poor. Sufficient quantities for sequencing were obtained by pooling eluates from several maps. The amount of tryptic digest that could be applied

	FLAGELLIN	FRAGMENT A	FRAGMENT B	FRAGMENT D	'C,D' COMPLEX
No. of Arg Residues	12	1	6	2	4
No. of Arg Peptides	12	2	6	4	3
No. of Tyr Residues	7	4	0	1	2
No. of Tyr Peptides	7	6	0	2	1
Total no. of Peptides	36	22	14	8	8
Total Lys + Arg Residues	38	17	11	4	9

TABLE 1·4 Numbers of tryptic peptides containing certain amino acids in S. senftenberg (SL 736) g,s,t flagellin and its CNBr fragments.

to any one map was limited; excess material at the origin prevented proper resolution of the peptides.

Peptides resulting from digestion of CNBr B with thermolysin, pepsin or staphylococcal protease were also isolated in this way, as were tryptic peptides of CNBr D.

(ii) Amino acid composition and sequence of CNBr B peptides

The position of the g,s,t CNBr B tryptic peptides on the peptide map and their amino acid composition as determined by amino acid analysis are given in Table 1·5. Also included in the table are the sequences of corresponding tryptic peptides of S. typhimurium H1-i flagellin with very similar amino acid compositions, as determined by Joys and Rankis (1972). There is at least one difference between them; threonine in peptide 15 of g,s,t CNBr B is replaced by alanine in TP 22 of S. typhimurium flagellin.

The amino acid composition of the g,s,t CNBr B peptides correlates with the positions of the peptides on peptide maps (Figure 1·14). For example, peptides containing a higher proportion of hydrophobic amino acids, such as leucine and isoleucine, tended to run further chromatographically.

The subtractive Edman degradation technique was used to sequence the g,s,t CNBr B tryptic peptides. However difficulties were encountered, both as a result of insufficient quantities of peptide, and because of technical difficulties associated with the amino acid analyser. Although it was therefore not possible to determine the sequences of the tryptic peptides, the strong similarity between their amino acid compositions and those of corresponding peptides in S. typhimurium H1-i flagellin, led to the conclusion that their sequences may also be the same.

The position on the peptide map and amino acid composition of thermolytic, staphylococcal protease and peptic peptides of CNBr B are

PEPTIDE ON TRYPTIC MAP	AMINO ACID COMPOSITION	SEQUENCE NH_2.....COOH	TRYPTIC PEPTIDE (TP)
1	asp, glu_2, ileu, leu - arg	Leu-Asn-Glu-Ileu-Asp-Arg	18
2	asp_4, $thre_4$, ser_2, glu_2, gly_2, ala val, ileu, leu - lys		
3	asp_2, ser, glu_5, $ileu_2$ - arg		
4	asp_6, $thre_2$, ser_4, glu_4, gly_2, ala_2, val_2, leu_2 - lys		
7	asp_{2-3}, glu, gly, ala_4, ileu - arg	Asp-Asp-Ala-Ala-Gly-Gln-Ala-Ileu-Ala-Asn-Arg	3
10	asp, ser, glu, gly_3 - lys		
11	asp, ser, gly, ala, val, ileu - lys		
13	thre, ser, glu, gly, ala, leu - arg	Gly-Leu-Thre-Gln-Ala-Ser-Arg	19
14	asp, ser_4, glu_2, gly, ala, ileu, leu - arg		
15	asp, thre, ser, ileu - lys	Ileu-Asn-Ser-Ala-Lys	22
17	ser_2, gly, leu_2 - arg	Leu-Ser-Ser-Gly-Leu-Arg	24
19	asp, ser, glu, gly_2, ala - lys		
	S. senftenberg g,s,t CNBr B TRYPTIC PEPTIDES	* S. typhimurium TRYPTIC PEPTIDES	

TABLE 1·5 The position and amino acid composition of S. senftenberg g,s,t CNBr B tryptic peptides.

* The sequence of corresponding tryptic peptides of S. typhimurium flagellin, as determined by Joys and Rankis (1972), are included for comparison.

FIGURE 1·14

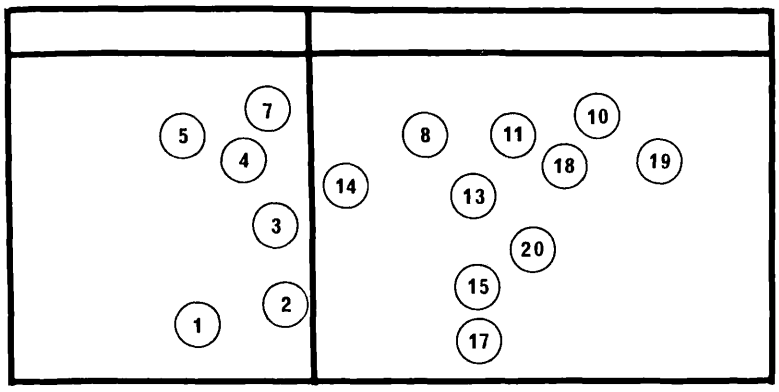
Enzymic peptide maps of CNBr B fragment of *S. senftenberg* (SL736) *g,s,t* flagellin: peptide reference numbers.

a Trypsin

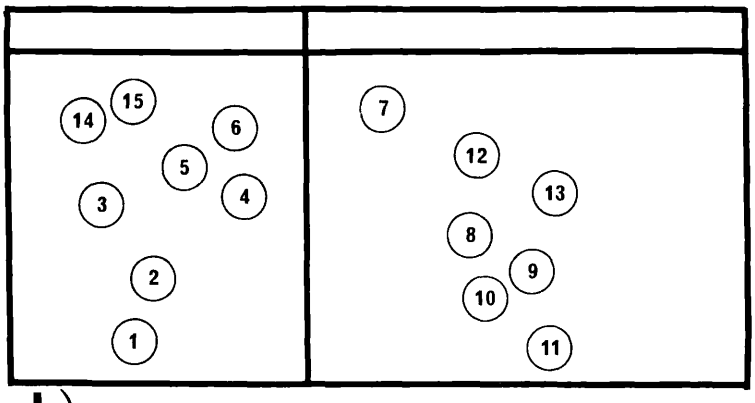
b Thermolysin

c Pepsin

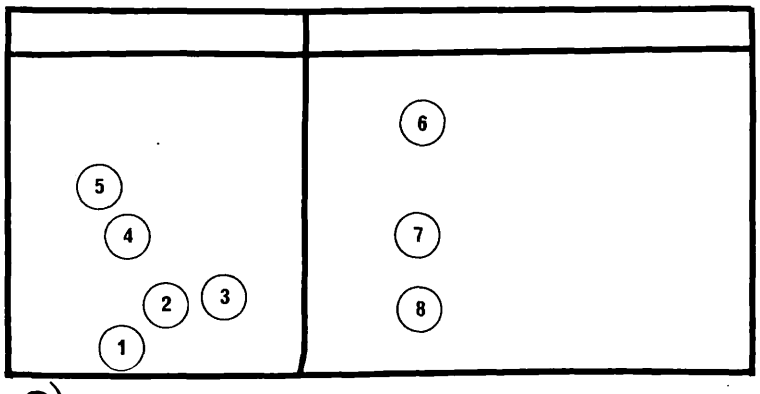
d Staphylococcal protease (V8)


7
5
4
3
2
1
8
11
10
18
19
14
13
20
15
17

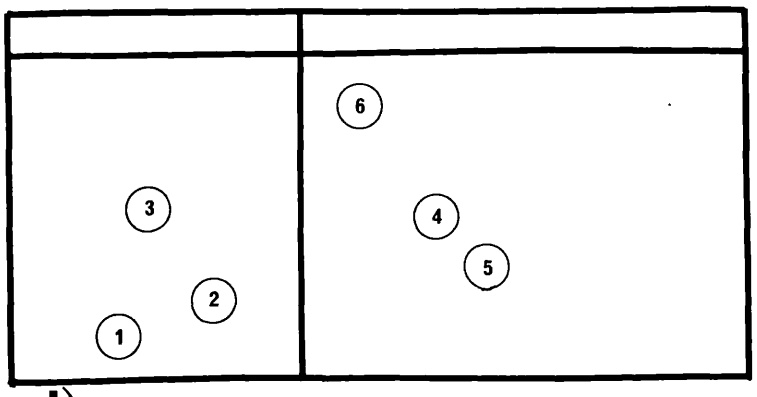
a)


15
14
6
5
4
3
2
1
7
12
13
8
9
10
11

b)


6
5
4
7
2
3
8
1

c)


6
3
4
5
2
1

d)

given in Tables 1·6, 1·7 and 1·8, respectively. Correspondingly numbered peptide maps are shown in Figure 1·14.

(iii) Amino acid composition of CNBr D peptides

The position on the peptide map and amino acid composition of tryptic peptides of g,s,t CNBr D are given in Table 1·9; the correspondingly numbered tryptic peptide map is shown in Figure 1·15. Peptide 1 has a very similar amino acid composition to that of TP 10 of S. typhimurium flagellin, except that isoleucine in peptide 1 is replaced by valine in TP 10.

The sequence of peptide 2 is obviously 'Ser - Arg'. Arginine must be the C-terminal amino acid, because trypsin cleaves on the carboxyl side of lysine and arginine residues. Peptide 2 corresponds to TP 26 in S. typhimurium flagellin.

PEPTIDE ON THERMOLYSIN MAP	AMINO ACID COMPOSITION
1	asp_{1-2}, glu, leu
2	ser, glu, gly, ala
3	asp_3, gly, ala
4	asp, ser, glu, gly
5	asp_{2-3}, gly, ala, lys
6	asp_2, glu, gly, ala_2, lys
8	asp_2, ser, ileu, leu_2, lys_2
9	asp, thre, gly, ala, leu_2, lys_2
10	$thre_2$, ser, glu, leu_2, lys, arg
11	leu_2, arg_2
12	asp_2, thre, ser, gly, ala, arg
14	asp_3, ser_2
15	asp_2, thre, ser

TABLE 1·6 The position and amino acid composition of thermolytic peptides of CNBr B fragment of S. senftenberg g,s,t flagellin.

PEPTIDE ON V8 MAP	AMINO ACID COMPOSITION
2	asp, glu_3, ileu, leu, arg
3	asp_2, thre, ser, glu
4	asp, glu, val, ileu, arg
5	asp_2, glu, gly, ileu, leu

TABLE 1·7 The position and amino acid composition of V8 (staphylococcal protease) peptides of CNBr B fragment of S. senftenberg g,s,t flagellin.

PEPTIDE ON PEPTIC MAP	AMINO ACID COMPOSITION
1	asp_4, glu_2, ileu, leu
2	asp, thre, ser, glu_2, leu
3	asp, glu_2, ileu
4	asp_3, thre, ser, gly
6	glu, arg
7	asp, ser_2, glu

TABLE 1·8 The position and amino acid composition of peptic peptides of CNBr B fragment of S. senftenberg g,s,t flagellin.

PEPTIDE ON TRYPTIC MAP	AMINO ACID COMPOSITION
1	asp_2, ser, glu, gly, ala, ileu, leu - arg
2	ser - arg
5	asp_3, $thre_2$, ser, gly, ala_2, ileu, leu - lys
6	asp, ser, glu, (gly), ala - arg

TABLE 1·9 The position and amino acid composition of tryptic peptides of CNBr D fragment of S. senftenberg g,s,t flagellin.

FIGURE 1·15

Tryptic peptide map of CNBr D fragment of S. senftenberg (SL 736) g,s,t flagellin: peptide reference numbers.

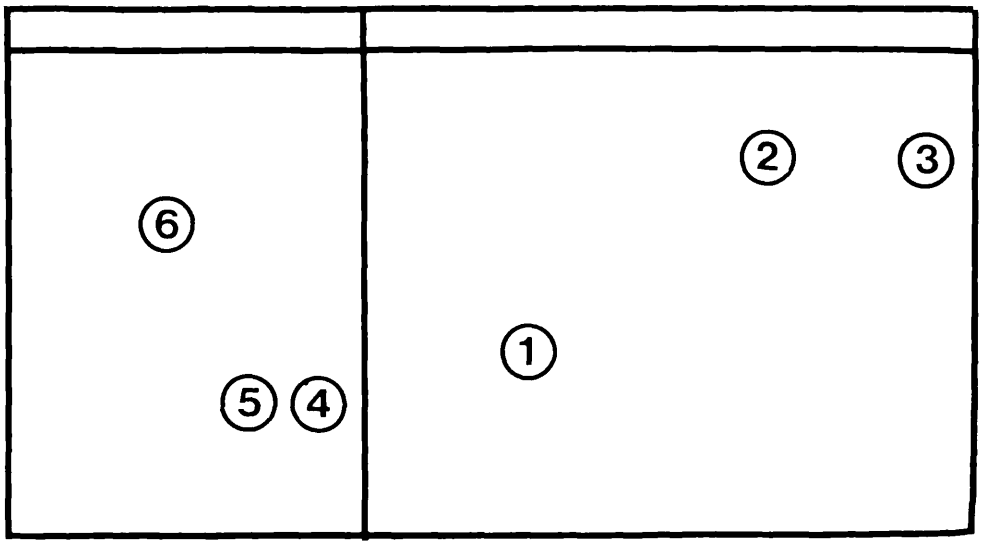


Figure 1·15

2. SEROLOGICAL DIFFERENCES BETWEEN FLAGELLA OF SALMONELLA g.... ANTIGENIC STRAINS

Antisera were prepared against S. senftenberg SL 736, H1-g,s,t; S. essen SL 588, H1-g,m and S. dublin SL 166, H1-g,p flagella. Single factor antisera were obtained by absorption of these antisera. The absorbing organisms used in each case are listed in Table 2.1.

FACTOR	ANTISERUM	ABSORBING CULTURE(S)
t	ser Senftenberg: g,s,t,z_8,(z_9)*	ser Essen: g,m + ser Montevideo: g,m,s
s	ser Senftenberg: g,s,t,z_8,(z_9)	ser Budapest: g,t,z_8,z_9 + ser Oranienberg: m,t_1,t_2,t_3
m	ser Essen: g,m	ser Dublin: g,p + ser Senftenberg: g,s,t,z_8,(z_9)
p	ser Dublin: g,p	ser Essen: g,m

TABLE 2.1 Preparation of anti-factor sera from Salmonella g.... antigenic strains.

* Presence unconfirmed

Thus the g antigenic factor was absorbed from the original serum in each case. The term "antigenic factor" refers to the antigenic unit detected by means of the cross-absorption-agglutination test. The g antigenic factor, however, can be further subdivided (Yamaguchi and Iino, 1969). The subfactors of the g antigenic factor have not yet been determined for the three antigenic types used for antiserum production. Although absorption was repeated until there was no further agglutination of the antiserum with the absorbing strain, it is possible that g subfactors not present in the absorbing strain were therefore not absorbed from the antiserum.

The presence of an antibody to the z_8 antigenic factor was implicated in the anti-g,m sera. After repeated absorption with bacteria of the g,p antigenic type, anti-g,m was still able to

agglutinate organisms of the g,q; g,t,z_8,z_9; g,s,t,z_8,(z_9) and f,g,z_8 antigenic types. The antigenic factor z_8 has been reported in all these strains except that of the g,q antigenic type. However, this does not exclude the possibility that z_8 is present in the g,q strain. After subsequent reabsorption of the anti-g,m sera with g,s,t,z_8,(z_9) bacteria, no agglutination with the four antigenic bacterial strains was detected. It was concluded that the z_8 antigenic factor was part of the antigenic determinant of the g,m antigenic strain.

The homologous agglutination titres of the unabsorbed and single factor antisera, expressed as the reciprocal of the highest dilution showing activity, were determined by slide agglutination (Table 2·2).

	ANTISERUM	ANTIGEN	AGGLUTINATION TITRE
G	Anti-g,s,t,z_8,(z_9)	g,s,t,z_8,(z_9)	8,192
H	Anti-g,m	g,m	8,192
J	Anti-g,p	g,p	4,096
S	Anti-s	g,s,t,z_8,(z_9)	2,048
T	Anti-t	g,s,t,z_8,(z_9)	512
M	Anti-m	g,m	512
P	Anti-p	g,p	128

TABLE 2·2 Agglutination titres of unabsorbed and single factor antisera.

All apparently negative reactions were checked under the low power objective of the microscope for weak agglutination. As would be expected, the single factor antisera had lower agglutination titres than the unabsorbed antisera from which they were obtained.

When the single factor antisera (1:10) were reacted with a range of g.... antigens, they agglutinated only those cells carrying the corresponding antigenic factor, and were therefore assumed to be specific for that particular antigenic factor.

The activity of the unabsorbed and single factor antisera, and the

ability of flagella and flagellin CNBr fragment A to inhibit activity, were tested by two different techniques:

Immobilisation-inhibition assay

The ability of unabsorbed and single factor antisera to immobilise organisms with the corresponding flagellar antigen(s) were tested, the results being recorded in Table 2·3.

	ANTISERUM	MOTILE CULTURES	IMMOBILISATION TITRE
un-absorbed	G	g,s,t	2,048
	H	g,m	1,024
	J	g,p	8,192
single factor	S	g,s,t	1,024
	T	g,s,t	2,048
	M	g,m	256
	P	g,p	128

TABLE 2·3 Immobilisation titres of unabsorbed and single factor antisera

Immobilisation titres are expressed as the reciprocal of the dilution at which there is approximately 80% inhibition.

In contrast to the agglutination titre, the immobilisation titre of anti-g,p (J) was higher than that of the two other unabsorbed antisera (G and H). In addition, the immobilisation titres of anti-s (S) and anti-t (T) were higher than would be expected from that of the unabsorbed serum (G).

The ability of flagella and flagellin CNBr fragment A to inhibit the immobilising activity of the antisera is presented in Table 2·4. The values given represent the concentration of the substance being tested (inhibitor) that just neutralised the immobilising activity of a dilution of antiserum which gave approximately 80% immobilisation. The immobilising activity of anti-g,s,t serum (G) was very effectively inhibited by g,s,t flagella. Slightly more g,s,t, CNBr A was required

ANTISERUM	g,s,t FLAGELLA	g,m FLAGELLA	g,p FLAGELLA	g,s,t CNBr A	g,m CNBr A	g,p CNBr A	g,p B	g,p D
G	0.32	ND	ND	2.56	2.56	2.56	ND	ND
H	ND	0.32	ND	250	1.28	250	ND	ND
J	ND	ND	250	250	250	250	500	500
S	2.56	ND	ND	5.12	ND	ND	ND	ND
T	5.12	ND	ND	5.12	250	250	ND	ND
M	ND	2.56	ND	40.96	5.12	5.12	ND	ND
P	ND	ND	5.12	250	250	2.56	ND	ND

Quantity of inhibitor required for inhibition (μg/0.25ml)

TABLE 2.4 Ability of flagella and flagellin CNBr fragments to inhibit the activity of unabsorbed and single factor antisera, as tested by the immobilisation technique.

ND Not determined

to inhibit activity than flagella; g,m CNBr A and g,p CNBr A were equally as effective as inhibitors as g,s,t CNBr A. As the three g.... antigenic types probably have g subfactors in common, these results are not surprising. However, anti-g,m serum (H), although effectively inhibited by g,m CNBr A, was still active when more than 250µg of g,s,t CNBr A or g,p CNBr A were added (that is, approximately 250 times the amount of g,m CNBr A). Anti-g,p serum (J) was obviously present in too high a concentration, making the technique insensitive. However, the results indicated that g,p CNBr B and g,p CNBr D were less effective as inhibitors than the CNBr A fragments tested.

Anti-s serum (S) was inhibited by almost equal concentrations of g,s,t flagella and g,s,t CNBr A, although these were larger amounts than those required to inhibit anti-g,s,t. This was also true for anti-t serum (T). However, g,m CNBr A or g,p CNBr A, even when present in concentrations 100 times that of g,s,t CNBr A, did not inhibit the immobilising activity of anti-t. These results correlate with the fact that g,m CNBr A or g,p CNBr A do not carry a t antigenic factor.

Similarly, anti-p serum (P) was inhibited by g,p flagella and g,p CNBr A, but not by g,m CNBr A or g,s,t CNBr A, neither of which carry a p antigenic factor.

The results obtained for anti-m serum (M) were, on the other hand, rather surprising. Not only g,m flagella and g,m CNBr A were effective as inhibitors of immobilising activity, but g,p CNBr A and, to a lesser extent, g,s,t CNBr A also. Although the two latter CNBr A fragments do not carry an m antigenic factor, their conformation must be such that they can interact with and inhibit anti-m activity.

Complement fixation-inhibition

The ability of the unabsorbed and single factor antisera to fix complement was tested.

Table 2·5 shows the micro-complement fixation protocol and results

								CONTROLS			
REACTION MIXTURE	1	2	3	4	5	6	7	8	9	10	11
ANTI-g,m (1:200) ml	1	1	1	1	1	1	1	1	1	1	1
SALINE, ml	3	3	3	3	3	3	3	4	4	5	6
COMPLEMENT (1:25), ml	1	1	1	1	1	1	1	1	1	1	
ANTIGEN (g,m FLAGELLA) ml	1	1	1	1	1	1	1	-	1	-	-
µg ANTIGEN/ML	256	128	64	32	16	8	4	-	256	-	-
	16-18 HOURS, 2-4°C										
EA ($5x10^7$/ml), ml	1	1	1	1	1	1	1	1	1	1	1
	30 MINUTES, 37°C										
HAEMOLYSIS, OD 413nm	.49	.26	.19	.10	.10	.15	.40	.54	.54	.55	.05
C-FIXATION, ΔOD: CONTROL OD (AVERAGE) MINUS REACTION OD	.05	.28	.35	.44	.44	.39	.14				
% C-FIXATION (ΔOD/CONTROL OD) x 100	9	52	65	81	81	72	26				

TABLE 2·5 Micro-complement fixation protocol and results for anti-g,m/g,m flagella system.

EA = sensitised erythrocytes

for anti-g,m. Similar results were obtained for the other antisera; a suitable dilution of antiserum was determined in each case. The g,m complement fixation curve (Figure 2·1), complement fixation plotted against antigen concentration, resembles the quantitative precipitin reaction in that zones of excess antibody, equivalence and antigen excess inhibition are recognisable.

The dilution of antiserum which gave a complete complement fixation curve, with maximum fixation around 70-80%, was subsequently used for inhibition studies. The effectiveness of the g,s,t; g,m and g,p CNBr A fragments as inhibitors of the complement fixing activity of the unabsorbed and single factor antisera was investigated. The protocol and results for the inhibition of the g,m immune system by g,m CNBr A is presented in Table 2·6. The percentage inhibition values of the flagellin CNBr A fragments for the various antisera are given in Tables 2·7 - 2·11.

Anti-g,s,t (1:800) is 100% inhibited by g,s,t CNBr A at a concentration of 256µg/ml. At the same concentration, g,m CNBr A brings about 36% inhibition. The antigen concentration (g,s,t flagella) during inhibition was 8µg/ml.

However, anti-g,m (1:200) is 100% inhibited by g,m CNBr A at 64µg/ml, at which concentration, g,s,t CNBr A only causes 8% inhibition. The antigen concentration (g,m flagella) during inhibition was 16µg/ml.

Thus g,m CNBr A is a more effective inhibitor of anti-g,s,t than g,s,t CNBr A is of anti-g,m. Other g,s,t CNBr fragments, B and D, were also tested for their ability to inhibit anti-g,s,t activity. Surprisingly, CNBr B at equimolar concentrations appeared to be as effective $\tilde{\phi}$n inhibitor of anti-g,s,t as g,m CNBr A. CNBr D, on the other hand, was a very poor inhibitor.

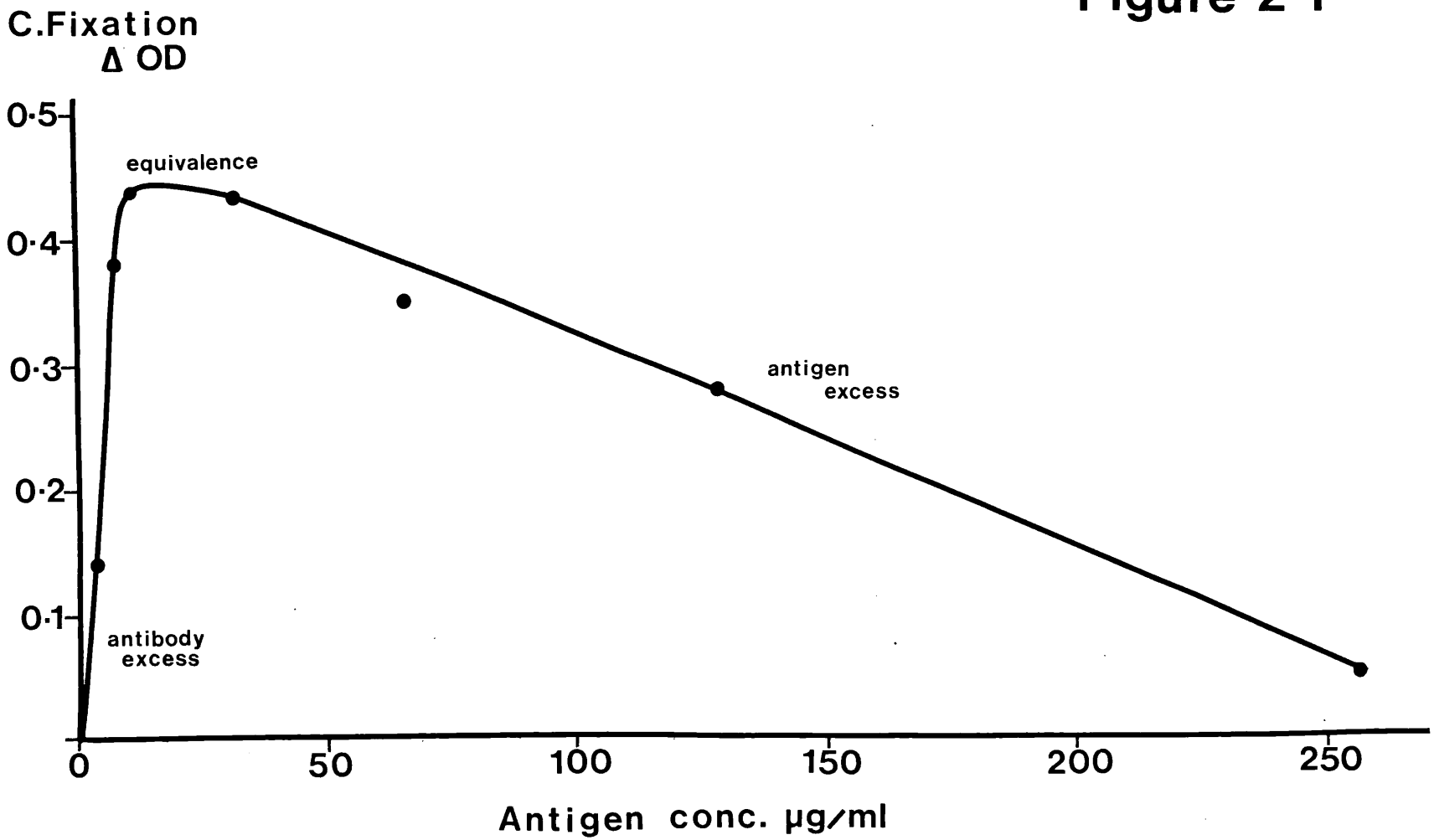
Both anti-t and anti-s were most effectively inhibited by g,s,t CNBr A; g,m and g,p CNBr A fragments were poorer inhibitors of anti-t

FIGURE 2·1

The S. essen g,m complement fixation curve: complement fixation vs. antigen concentration.

As in the quantitative precipitin reaction, zones of excess antibody, equivalence and antigen excess are recognisable.

Figure 2·1


C.Fixation
Δ OD
0·5
0·4
0·3
0·2
0·1
equivalence
antigen
excess
antibody
excess
0
50
100
150
200
250
Antigen conc. µg/ml

								CONTROLS				
REACTION MIXTURE	1	2	3	4	5	6	7	8	9	10	11	12
ANTI-g,m (1:200) ml	1	1	1	1	1	1	1	1	-	-	-	-
SALINE, ml	2	2	2	2	2	2	3	4	4	4	5	6
COMPLEMENT (1:25), ml	1	1	1	1	1	1	1	1	1	1	1	
g,m CNBr A, ml	1	1	1	1	1	1	-	-	-	1	-	-
µg g,m CNBr A/ml	64	32	16	8	4	2	-					
g,m FLAGELLA (16µg/ml)	1	1	1	1	1	1	1	-	1	-	-	-
				16-18 hours, 2-4°C								
EA ($5x10^7$/ml), ml	1	1	1	1	1	1	1	1	1	1	1	1
				30 minutes, 37°C								
HAEMOLYSIS, OD 413nm	.58	.56	.48	.43	.16	.14	.10	.60	.56	.58	.55	.04
C·FIXATION: AVERAGE OF OD VALUES OF C, Ab, Ag AND I CONTROLS MINUS REACTION OD	0	.013	.093	.143	.413	.433	.473					
% INHIBITION	100	97	80	70	13	9	0					

TABLE 2·6 Inhibition of g,m immune system by g,m CNBr A

where $$\% \text{ Inhibition} = \left[1 - \frac{\text{C·Fixed in presence of inhibitor}}{\text{C·Fixed in absence of inhibitor}}\right] \times 100$$

C = complement
EA = sensitised erythrocytes

INHIBITOR µg/ml	% INHIBITION g,s,t CNBr A	g,m CNBr A
1024	100	90
512	100	79
256	100	36
128	95	17
64	86	17
32	86	4
16	43	2
8	14	2
4	10	2
2	7	0
1	0	0
0	0	0

TABLE 2·7a Inhibition of anti-g,s,t (1:800) by g,s,t CNBr A and g,m CNBr A.

Antigen concentration = 8µg/ml.

INHIBITOR µg/ml	% INHIBITION g,s,t CNBr B	g,s,t CNBr D
12,800	95	30
1,280	95	7
128	33	0

TABLE 2·7b Inhibition of anti-g,s,t (1:800) by equimolar concentrations of g,s,t, CNBr B and CNBr D.

Antigen concentration = 8µg/ml.

INHIBITOR µg/ml	% INHIBITION g,s,t CNBr A	g,m CNBr A
256	43	100
128	32	100
64	8	100
32	4	97
16	4	80
8	4	70
4	4	13
2	0	9
1	0	6
0	0	0

TABLE 2.8 Inhibition of anti-g,m (1:200) by g,s,t CNBr A and g,m CNBr A.

Antigen concentration = 16µg/ml.

INHIBITOR µg/ml	% INHIBITION g,s,t CNBr A	INHIBITOR µg/ml	% INHIBITION g,m CNBr A	g,p CNBr A
8	59	1,000	52	35
4	42	100	19	4
2	42	10	4	0
1	27			
0.5	0			
0	0			

TABLE 2.9 Inhibition of anti-t (1:200) by g,s,t; g,m and g,p CNBr A fragments.

Antigen concentration = 4µg/ml.

INHIBITOR µg/ml	% INHIBITION g,s,t CNBr A	INHIBITOR µg/ml	% INHIBITION g,m CNBr A
256	59	1,000	46
128	21	100	15
64	21	10	5
32	5	0	0
16	5		
0	0		

TABLE 2-10 Inhibition of anti-s (1:300) by g,s,t and g,m CNBr A fragments.

Antigen concentration = 32µg/ml.

INHIBITOR µg/ml	% INHIBITION g,p CNBr A	INHIBITOR µg/ml	% INHIBITION g,s,t CNBr A	% INHIBITION g,m CNBr A
16	0	1,000	0	0
8	0	100	0	0
4	0	10	0	0
2	0			
1	0			
0.5	0			
0	0			

TABLE 2-11 Inhibition of anti-p (1:150) by g,p; g,s,t and g,m CNBr A fragments.

Antigen concentration = 2µg/ml.

than g,m CNBr A was of anti-s.

Anti-p was not inhibited by g,p; g,m or g,s,t CNBr A fragments at the concentrations tested. However, it is possible that g,p CNBr A would have inhibited activity if higher concentrations had been used.

Sequence basis of the serological differences between Salmonella g.... antigenic flagellins.

i) Amino acid composition of CNBr A fragments of g.... antigenic flagellins.

In Table 2·12 are presented the amino acid compositions of the CNBr A fragments of g,s,t; g,m and g,p flagellins. For comparison, the amino acid composition of the CNBr A fragment of S. adelaide f,g flagellin (Parish and Ada, 1969) was included in the table.

The amino acid contents of the g,s,t; g,m and g,p fragments appear very similar; because of the size of the peptides, it is unlikely that small composition differences would be detected unless they involved amino acids present in low amounts. However, possible differences between the composition of these fragments and that of f,g CNBr A are indicated; the alanine content of g,s,t CNBr A, and the serine content of g,m CNBr A were significantly higher than the corresponding values of f,g CNBr A.

ii) Enzymic digestion of g.... antigenic flagellins and their CNBr A fragments.

Salmonella g,s,t; g,m; g,p and f,g flagellins were digested with each of five enzymes, trypsin, chymotrypsin, thermolysin, pepsin and papain, and peptide maps prepared. Papain digests of the flagellins gave maps with a similar pattern of spots in each case (Figure 2·2). This was also true for tryptic and peptic digests of the flagellins (Figure 1·8). Tests specific for tyrosine and arginine residues carried out on tryptic peptide maps of the flagellins showed a similar distribution of tyrosine- and arginine-containing peptides in the g,s,t;

AMINO ACID	g,s,t CNBr A	g,m CNBr A	g,p CNBr A	*f,g CNBr A
Asp	34	33	34	32
Thre	21	21	21	22
Ser	10	11	10	8
Hse	0.8	0.7	0.6	1
Glu	11	12	11	11
Pro	2	2	2	2
Gly	18	18	18	18
Ala	25	24	23	22
Val	14	14	15	16
Met	0	0	0	0
Ileu	7	8	9	8
Leu	9	8	8	8
Tyr	4	3	4	5
Phe	4	4	4	5
His	0	0	0	0
Lys	16	16	15	17
Arg	1	1	1	1
TOTAL	177	177	176	176

TABLE 2-12 Amino acid analysis of CNBr A fragments of Salmonella g.... antigenic flagellins.

Hse denotes homoserine.

* Values obtained by Parish and Ada (1969).

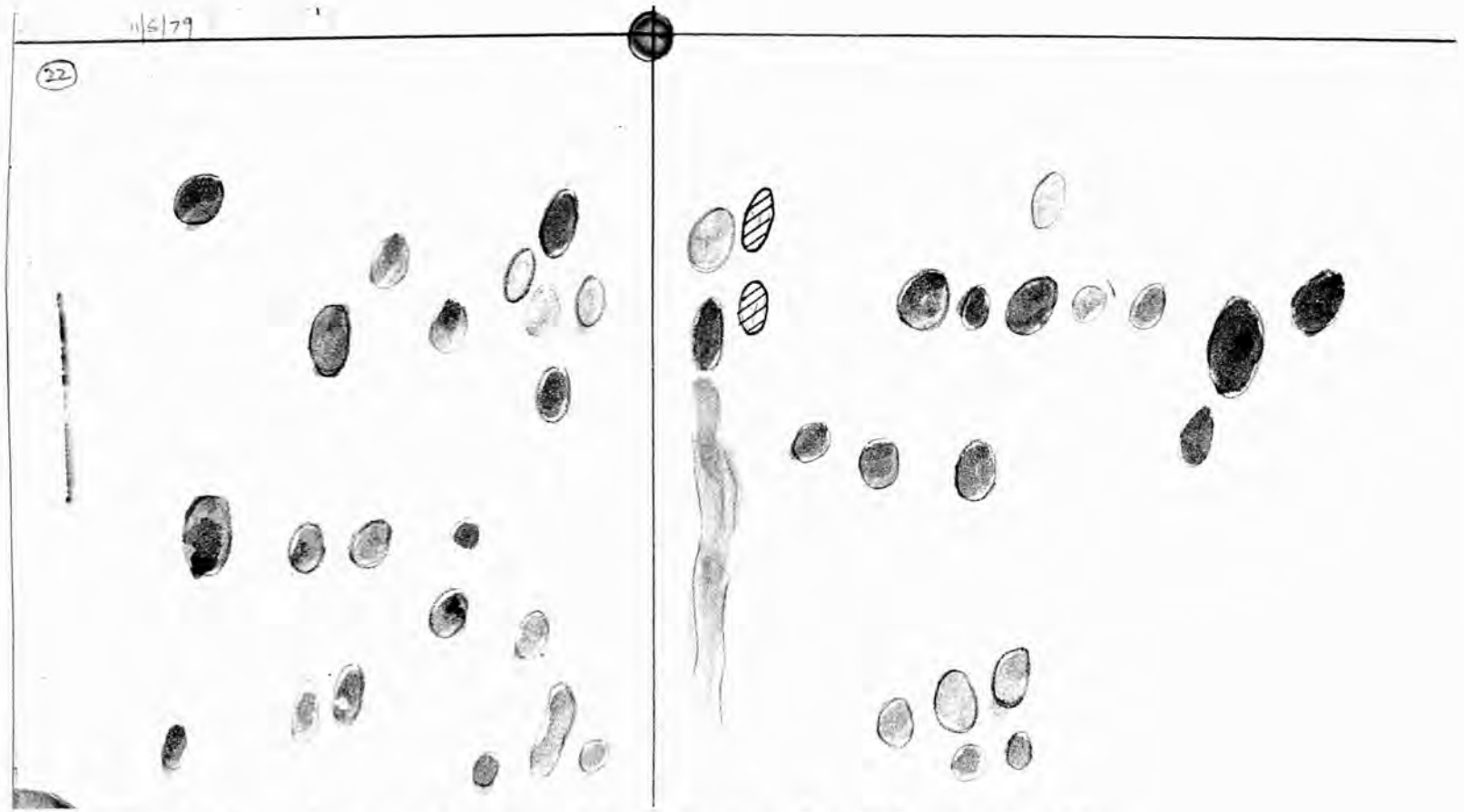


FIGURE 2·2 Papain peptide map of S. senftenberg (SL 736) g,s,t flagellin.

- peptide with N-terminal glycine or amide residue

g,m and f,g flagellins. However, a comparison of the chymotrypsin peptide maps revealed a possible peptide difference; a basic peptide in the g,s,t map had moved much more slowly chromatographically than a corresponding peptide in the g,m; g,p and f,g maps (Figure 2·3). A peptide difference was also detected in the thermolysin peptide maps; a peptide present in the g,s,t and f,g maps was absent in the maps of g,m and g,p flagellins (Figure 2·4).

It is of note, however, that a peptide map shows slight variations in its pattern of spots from run to run, so that care must be taken to ascertain the significance of any possible peptide differences. To achieve this, thermolytic digests of g,s,t; g,m; g,p and f,g flagellins were fractionated into acidic, neutral and basic peptides by paper electrophoresis at pH 6.5. The fractionated peptides were eluted, concentrated, spotted onto chromatography paper and subjected to descending chromatography, followed by electrophoresis at pH 1.9 (neutral peptides) or pH 6.5 (acidic, and basic peptides).

Comparison of the peptide maps of the basic peptides, and of the peptide maps of the neutral peptides, from the four antigenic types, confirmed the presence of peptide differences (Figure 2·5). A strongly staining peptide was detected in peptide maps of the neutral peptides of g,s,t and f,g flagellins, but was absent from those of g,m and g,p flagellins. In addition, a weaker staining peptide present in g,s,t and g,m (double spot), was absent in g,p and f,g flagellins. The peptide maps of basic peptides revealed a strongly staining peptide in g,m; g,p and f,g flagellins which was missing from g,s,t flagellin. This peptide difference had not been observed on the whole flagellin thermolysin maps.

Comparison of the peptide maps of acidic peptides showed no apparent peptide differences (Figure 2·5).

Serological studies (Parish and Ada, 1969, Ichiki and Parish, 1972)

FIGURE 2·3

Chymotryptic peptide map of Salmonella g.... antigenic flagellins (only relevant peptides shown).

A - peptide present in g,s,t, absent in g,m; g,p and f,g flagell:

B - peptide absent in g,s,t, present in g,m; g,p and f,g flagell:

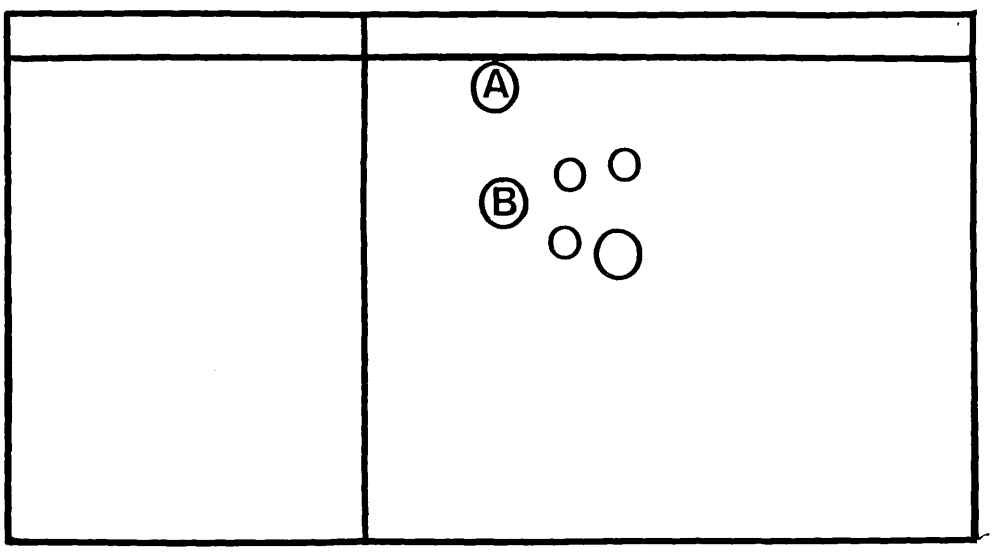


Figure 2-3

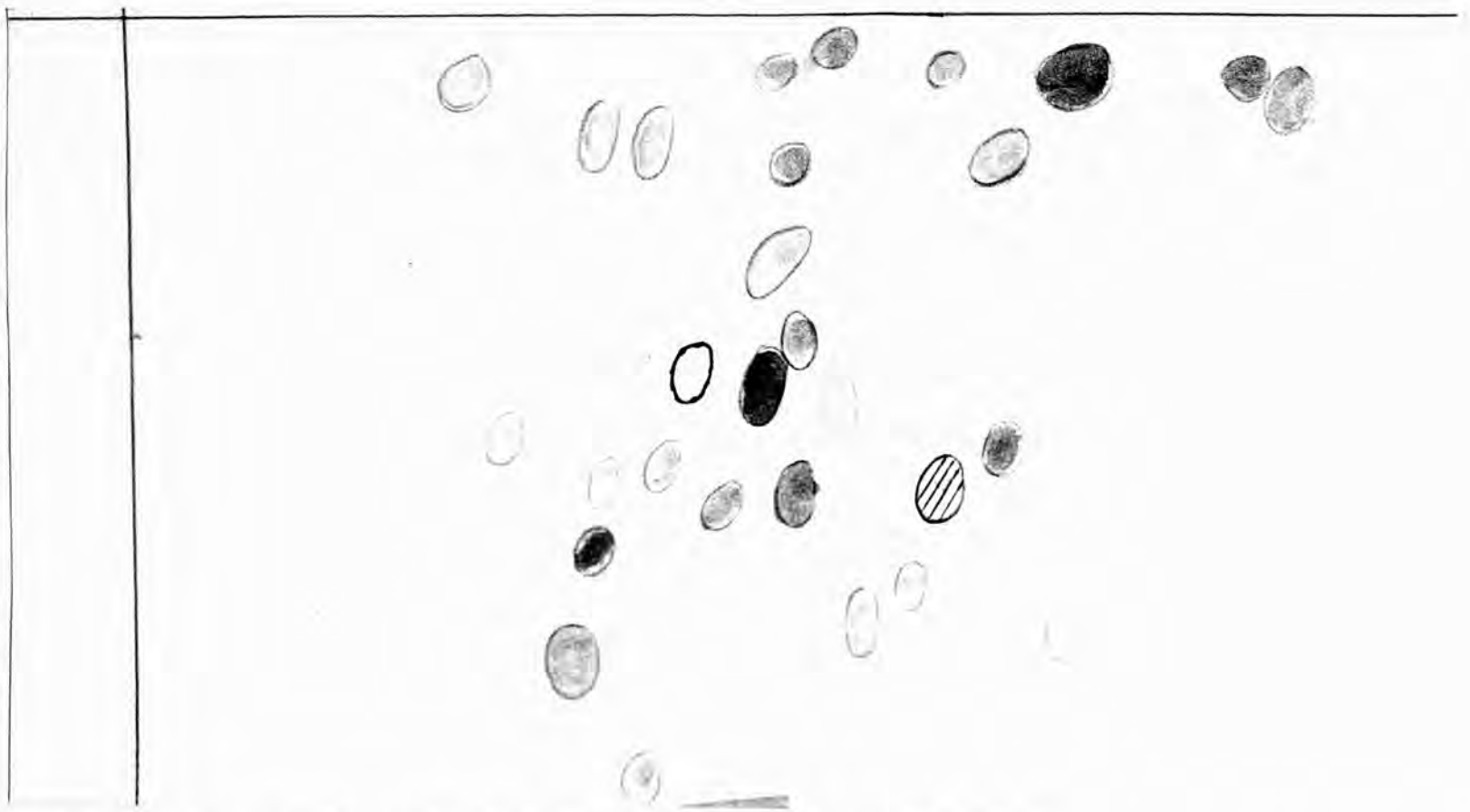


FIGURE 2·4 Thermolytic peptide map of Salmonella g.... antigenic flagellins

Unshaded spot denotes peptide present in g,s,t and f,g, but absent in g,m and g,p flagellins.

 - peptide with N-terminal glycine or amide residue.

FIGURE 2·5

Thermolytic peptide maps of Salmonella g.... antigenic flagellins

a) Neutral peptides (pH 1.9 electrophoresis)

Upper unshaded spot denotes a strongly staining peptide present in g,s,t and f,g, but absent in g,m and f,p flagellins.

Lower unshaded spot denotes a weaker staining peptide present in g,s,t and g,m (double spot), but absent in g,p and f,g flagellins.

b) Basic peptides (pH 6.5 electrophoresis)

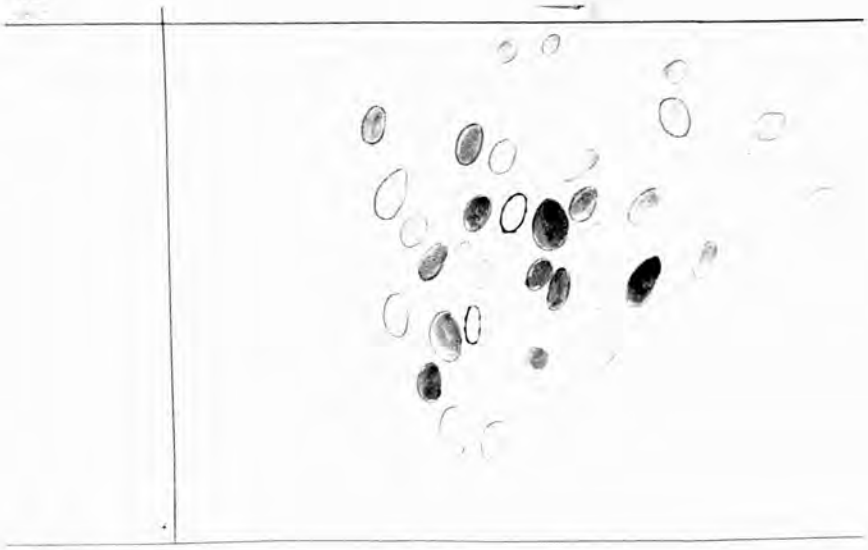
Unshaded spot denotes a strongly staining peptide present in g,m; g,p and f,g flagellins, but missing from g,s,t flagellin.

c) Acidic peptides (pH 6.5 electrophoresis)

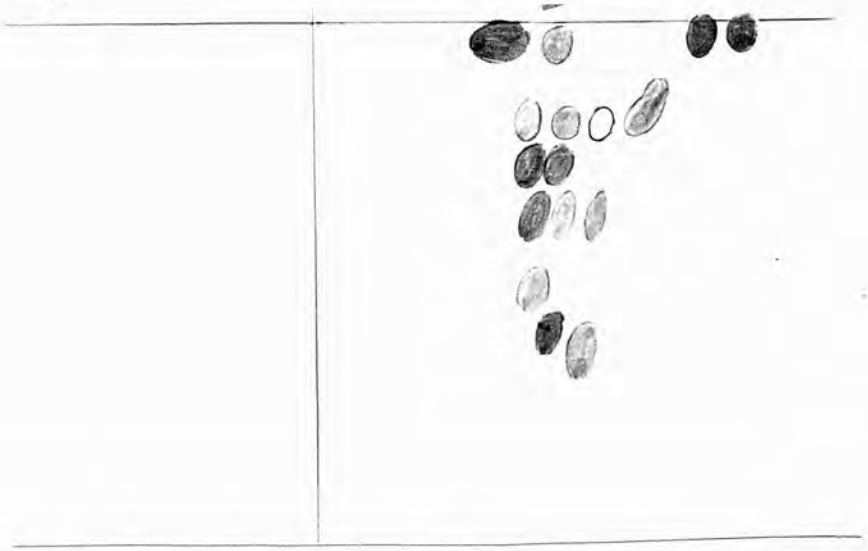
No peptide differences.

 - peptide with N-terminal glycine or amide residue.

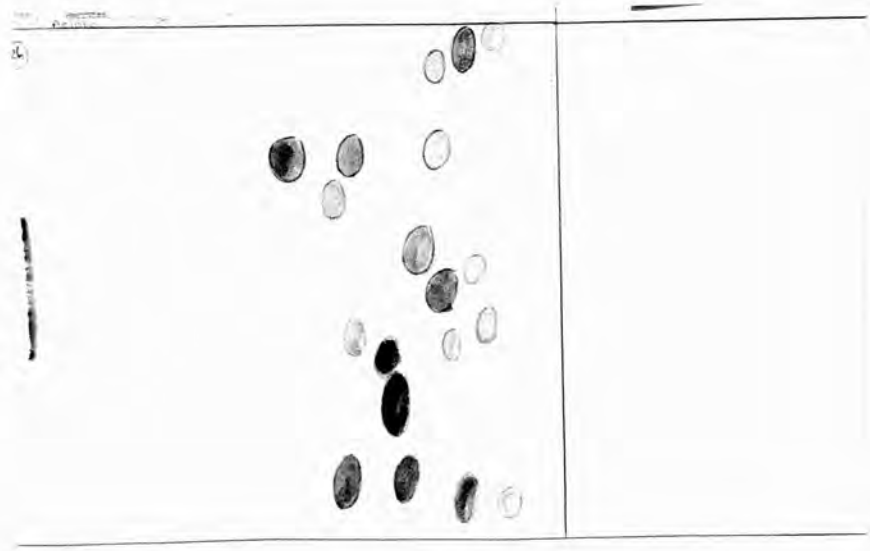
a



b



c



have indicated that CNBr fragment A of flagellin is most probably the major antigenic component of the molecule. Thus any amino acid differences responsible for antigenic variation would be expected to reside in the CNBr A fragment of the flagellin molecule. The neutral peptide difference (but not the basic peptide difference) was detected on thermolysin peptide maps of the whole CNBr A fragments as anticipated. (There was insufficient f,g CNBr A fragment for these studies.) The basic peptide difference was also detected when the CNBr A fragments were fractionated into acidic, neutral and basic peptides, as described for the whole flagellins. The weakly staining neutral peptide present on g,s,t and g,m flagellin maps, was absent from the peptide maps of the neutral peptides isolated from their corresponding CNBr A fragments. The chymotryptic peptide difference present in g,s,t flagellin was also confirmed and located in the CNBr A fragment.

The amino acid substitutions responsible for the peptide differences between the g.... antigenic flagellins have not yet been identified.

iii) Cleavage of g,s,t CNBr A fragment

Preliminary work was carried out to cleave the CNBr A fragment of g,s,t flagellin into smaller peptides, both to locate the antigenic site more precisely, and to facilitate sequencing studies. Amino acid analysis of CNBr A fragment suggested the presence of only one arginine residue (for a molecular weight of 19,000). Therefore, it should be possible to break the fragment into two pieces by limiting tryptic digestion to the arginine residue. Obviously the location of the arginine residue will determine the relative sizes of the two resulting fragments; if the residue is at one end of the CNBr A fragment, a large and a small fragment would be formed. Trifluoroacetylation and citraconylation were employed to modify the lysine residues of CNBr A fragment, thus preventing tryptic cleavage at these residues. Modified

tryptic digests were subjected to polyacrylamide gel electrophoresis at pH 2.7 in 8M urea; the results were inconclusive.

An alternative approach was the use of limited enzyme digestion in suboptimal conditions. Digestion of CNBr fragment A with pepsin for one hour at suboptimal pH, and subsequent polyacrylamide gel electrophoresis at pH 2.7 in 8M urea, revealed a strong double band plus one weak band running in a position between that of CNBr A and CNBr B fragments (Figure 2·6). These bands represent a region of the CNBr A fragment resistant to the action of pepsin.

Digestion with trypsin, even at the optimal temperature of 37°C, left an undigested core represented by two bands with very similar electrophoretic mobilities running faster than CNBr A on pH 2.7 polyacrylamide gels, in 8M urea. Similar results were obtained when whole g,s,t flagellin (Figure 2·7), g,m CNBr A and g,p CNBr A were treated in the same manner. An attempt to separate the two peptides on Sephadex columns was unsuccessful; ion-exchange chromatography might prove to be useful in this respect.

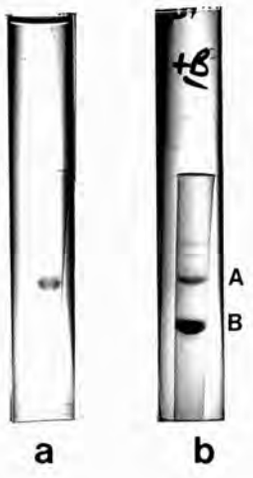


FIGURE 2·6

pH 2.7 polyacrylamide gel electrophoresis of CNBr fragments of S. senftenberg g,s,t flagellin, in 8M urea.

a) CNBr A digested with pepsin for one hour at suboptimal pH.

b) Mixture of CNBr A and CNBr B fragments, undigested.

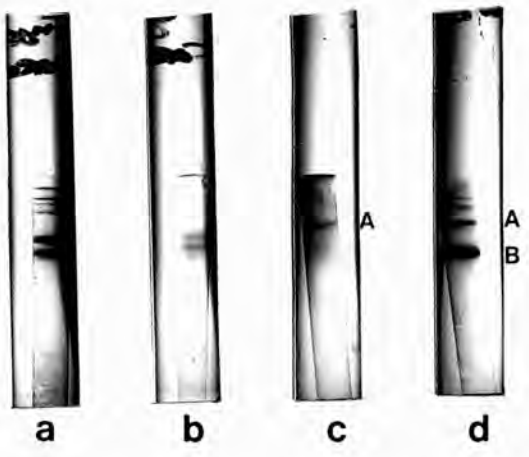


FIGURE 2·7

pH 2.7 polyacrylamide gel electrophoresis of S. senftenberg g,s,t flagellin and its CNBr fragments, in 8M urea.

a) Flagellin tryptic core.

b) CNBr A tryptic core.

c) CNBr A, undigested.

d) Mixture of CNBr A and CNBr B fragments, undigested.

3. MORPHOLOGICAL MUTANTS OF SALMONELLA g.... ANTIGENIC STRAINS

a) Enzymic digestion of mutant flagellins

The morphological mutants of g,s,t; g,m and f,g antigenic strains, together with the waveform of their flagella, are presented in Table 3·1.

Trypsin

Initially the g,s,t; g,m and f,g mutant flagellins were digested with trypsin. Comparison of the resulting peptide maps with that of their corresponding wild-type flagellin revealed no significant differences in peptide pattern.

Tests for the presence of tyrosine and arginine residues in the tryptic peptides of g,s,t M5-2 and M55-3; g,m D8-1 and f,g D24-2 flagellins were carried out on the peptide maps. The distribution of tyrosine- and arginine-containing peptides appeared the same in the g,s,t and g,m mutant flagellins as in their corresponding wild-type flagellin (and in the other wild-type flagellin, also). However, a tyrosine-containing peptide in f,g D24-2 flagellin moved much more slowly chromatographically than the corresponding peptide in the wild-type f,g flagellin (Figure 3·1). The same peptide in wild-type g,s,t flagellin had been located in CNBr fragment D (Figure 1·13).

Thermolysin

As the more specific enzyme trypsin had failed to reveal the peptide differences associated with the variety of waveform of the mutant flagella, a less specific enzyme, thermolysin, was employed, which resulted in a larger number of peptides on the peptide maps. Comparison of the thermolytic maps of the g,s,t and g,m mutant flagellins with that of their corresponding wild-type flagellin revealed no significant peptide differences, except in the case of g,s,t M54-2 flagellin (Figure 3·2). When the thermolytic digest of the mutant flagellin was spotted onto chromatography paper and subjected to

S. senftenberg SL 736 g,s,t		S. derby SL 175 f,g		S. essen SL 588 g,m	
NUMBER	WAVEFORM	NUMBER	WAVEFORM	NUMBER	WAVEFORM
M1-1	CURLY II	M2-2	CURLY II	D2-2	CURLY II
M5-2	CURLY IV	M5-1	"	D9-1	"
M32-2	CURLY II	M8-2	"	D9-2	"
M51-4	POLYMORPHIC	M9-1	"	D6-1	POLYMORPHIC
M53-1	CURLY II	M10-1	"	D10-1	"
M54-2	"	M13-1	"	D11-2	"
M55-3	"	D24-2	"	D8-1	STRAIGHT
D10-2	"	M6-2	STRAIGHT		

TABLE 3·1 Flagella waveform of morphological mutants of Salmonella g.... antigenic strains. (Hogben, personal communication.)

FIGURE 3.1

Tyrosine-containing peptides in a tryptic peptide map of f,g D24-2 flagellin (only relevant peptides shown).

A - peptide present in mutant, absent in f,g wild-type flagellin.

B - peptide absent in mutant, present in f,g wild-type flagellin.

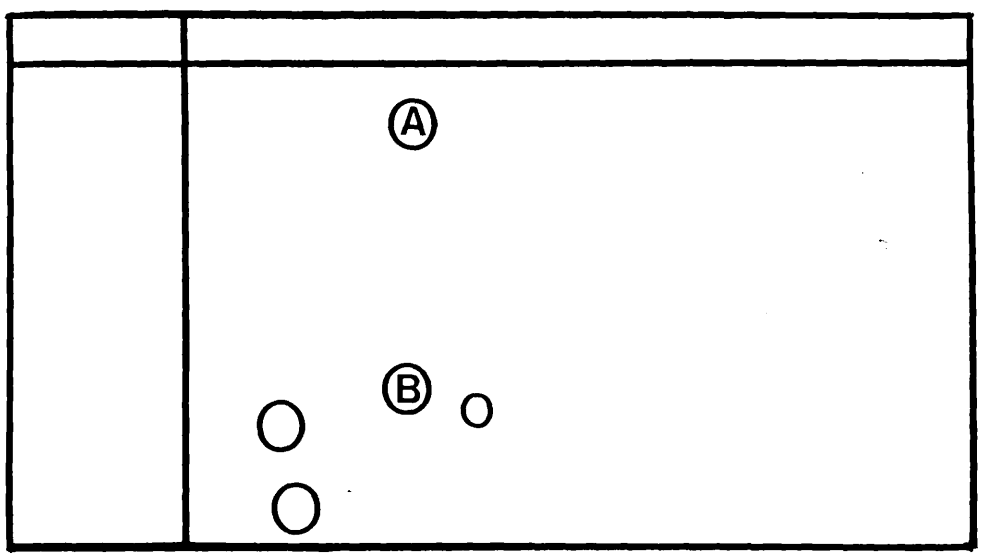


Figure 3·1

FIGURE 3·2

Thermolytic peptide map of g,s,t M54-2 flagellin (only relevant peptides shown).

A - peptide absent in mutant, present in g,s,t wild-type flagellin.

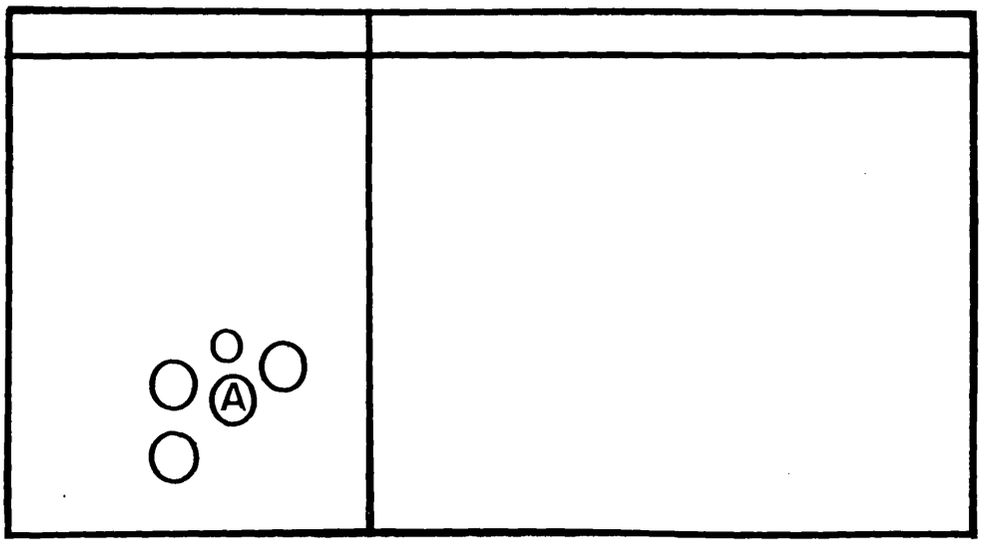


Figure 3·2

descending chromatography for 24 hours (instead of the usual 17 hours), followed by electrophoresis at pH 6.5, an acidic peptide present in the wild-type g,s,t flagellin was shown to be absent. No replacement peptide was detected in the mutant flagellin. Thermolytic peptide maps of CNBr fragments of g,s,t mutant flagellins suggest that the peptide is located in CNBr B or D.

Unexpectedly, the peptide was also absent from flagellin thermolytic peptide maps of "revertants" of the g,s,t M54-2 mutant strain. Either these are not true revertants, or the peptide difference is not correlated with the change in waveform of the flagella.

The wild-type peptide was eluted and subjected to quantitative amino acid analysis; a residue each of aspartic, serine, alanine and phenylalanine was present. Thermolysin preferentially hydrolyses the bond in which the NH_2 group is donated by a hydrophobic amino acid. This phenylalanine is probably the N-terminal amino acid of the peptide. The amino acid composition of the peptide correlates well with its electrophoretic (moves towards the anode) and chromatographic (moves fast) behaviour.

Preliminary work involving the fractionation of the mutant thermolytic peptides into acidic, basic and neutral types by pH 6.5 paper electrophoresis has, so far, revealed no peptide differences.

Pepsin

A peptide difference was also observed in the g,s,t M54-2 flagellin when its peptic peptide map was compared to that of the wild-type (Figure 3.3). (A longer time for chromatography was used, as for the thermolysin map.) A peptide present in the wild-type flagellin moved much faster chromatographically in the mutant flagellin. When the peptide from the wild-type flagellin and its counterpart in the mutant flagellin were eluted, hydrolysed and subjected to quantitative amino acid analysis, a single amino acid difference was detected. The wild-

FIGURE 3·3

Peptic peptide map of g,s,t M54-2 flagellin (only relevant peptides shown).

A - peptide absent in mutant, present in wild-type g,s,t flagellin.

B and C - peptides present in mutant, absent in wild-type g,s,t flagellin.

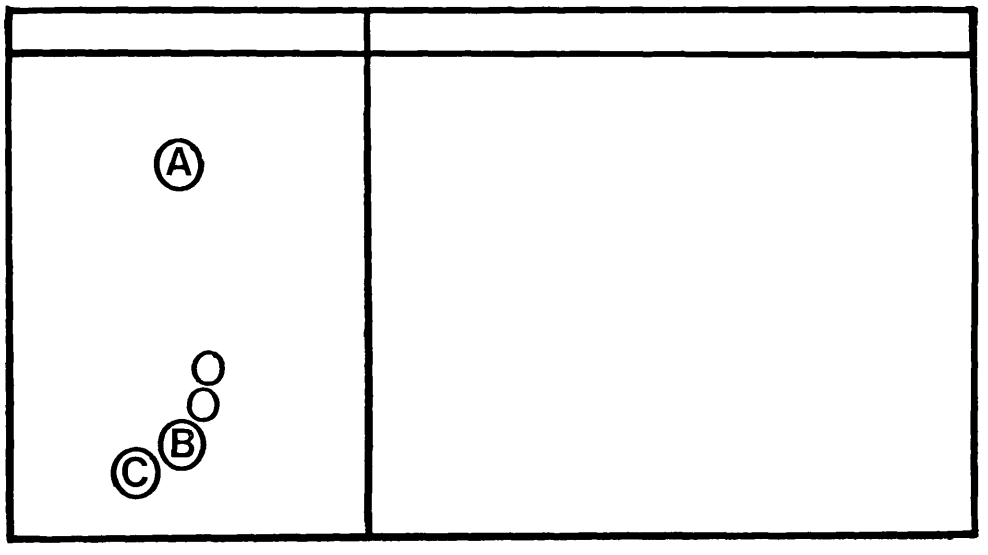


Figure 3-3

type peptide consisted of a residue each of aspartic acid, serine and alanine; serine was replaced by leucine in the mutant peptide. Because the replacement amino acid in the mutant peptide was more hydrophobic than the corresponding amino acid in the wild-type peptide, the mutant peptide moved faster chromatographically.

Sometimes two spots were observed instead of one when the mutant peptide map was repeated. The slightly slower running spot consisted of aspartic acid, leucine and alanine, and the electrophoretically faster one of aspartic acid and leucine only, suggesting that insertion of leucine created a further cleavage site for pepsin.

Assuming a single base change, a transition from C to U in the second base of serine (UC_G^A) has taken place.

b) Cyanogen bromide digestion of mutant flagellins

As peptide differences are more difficult to locate in enzymic peptide maps of the whole flagellin, g,s,t mutant flagellins were digested with cyanogen bromide to break up the protein into more manageable pieces. Previous results had indicated that any amino acid differences present were more likely to be located in CNBr fragments B, C or D than in CNBr A.

Comparison of tryptic peptide maps of the CNBr B, C and D fragments of the mutant flagellins with those of the wild-type revealed no apparent peptide differences.

Certain mutant CNBr fragments were also digested with thermolysin, staphylococcal protease or pepsin. However, again, the peptide maps of these digests showed no reproducible differences as compared to those of the wild-type flagellin fragments.

c) Amino acid composition of mutant flagellins

It would be impossible to detect a difference in the numbers of a certain amino acid residue by comparing the amino acid analysis of mutant and wild-type flagellins as the numbers involved are fairly

large. However, if the mutant flagellin possessed an amino acid that was absent from the wild-type flagellin, amino acid analysis could prove a useful tool in the search for amino acid differences. Just such an amino acid, histidine, was detected in g,s,t M32-2 and g,s,t D10-2 flagellins (Table 3.2).

The reason why two residues of histidine were present in M32-2 flagellin (a single amino acid substitution was anticipated) and why a higher proportion of proline was present in the mutant flagellins as compared to the wild-type, is not clear.

Amino acid analysis of g,s,t M55-3 flagellin showed an absence of histidine residues.

To try to locate the histidine-containing peptide(s) in the mutant flagellins, thermolytic digests were subjected to pH 1.9 paper electrophoresis followed by staining; the Pauly reaction was used. Any histidine-containing peptides stain pink; pink bands were observed in both the mutant and wild-type digests. As wild-type flagellin possesses no histidine residues, these results were obviously invalid. However, a fast-moving peptide(s) in g,s,t D10-2 flagellin also stained pink; the corresponding peptide(s) in the g,s,t wild-type and g,s,t M32-2 flagellins did not.

d) C-terminal analysis

Three mutant flagellins: g,s,t M5-2 (curly IV); g,m D8-1 (straight) and g,s,t M55-3 (curly II) were digested with carboxypeptidase B and the supernatants subjected to quantitative amino acid analysis. The same results were obtained as for the g,s,t and g,m wild-type flagellins (see Section 1d)).

Thus the C-terminal sequence for the mutant flagellins analysed is:

- Ser - Leu - Leu - ArgCOOH

AMINO ACID	g,s,t WILD-TYPE	g,s,t M32-2	g,s,t D10-2
Asp	68	64	65
Thre	34	32	36
Ser	34	36	35
Glu	37	39	39
Pro	3	6	10
Gly	31	31	34
Ala	48	48	48
Val	28	25	28
Met	3	3	3
Ileu	22	22	21
Leu	27	30	28
Tyr	7	6	6
Phe	10	11	9
His	0	2	1
Lys	26	26	25
Arg	12	13	13
TOTAL	390	394	401

TABLE 3•2 Amino acid analysis of S. senftenberg (SL 736) g,s,t wild-type and mutant flagellins.

4. AMINO ACID COMPOSITION OF PROTEUS FLAGELLINS

a) Total amino acid analysis

In Tables 4·1, 4·2, 4·3, 4·4 and 4·5 are presented the amino acid analyses of various Proteus flagellins. The values are calculated from analyses of 24 hour hydrolysate samples, assuming a molecular weight of 40,000.

All the flagellins have a high proportion of aspartic and glutamic acids (or amides) and alanine. Little tyrosine, phenylalanine, methionine or proline was present. Histidine was detected in certain strains of each Proteus species; cysteine or cysteic acid was always absent. The numbers of proline and methionine residues were fairly constant among the P. morganii flagellins, but they varied appreciably between the other strains studied.

The ratio of phenylalanine to tyrosine was greater than one in all the Proteus flagellins examined, and varied between 2-5:1.

Comparison of the amino acid analyses of Proteus flagellins with those of the Salmonella g.... antigenic flagellins (Section 1) reveals certain differences. For example, in the Proteus flagellins, the proportion of threonine and of serine residues is lower, and of glutamic acid residues is higher than in the Salmonella flagellins. There is variability in the numbers of proline and methionine residues between flagellins of different strains of P. vulgaris, of P. inconstans and of P. rettgerri, although the value obtained for methionine (and valine) in P. rettgerri NCTC 7475 flagellin is somewhat dubious. This variability is not observed between flagellins of P. morganii strains or between Salmonella g.... antigenic flagellins. Histidine is absent in the Salmonella g.... antigenic flagellins examined (except possibly g,q flagellin), whereas it is present in flagellins of several of the Proteus strains.

b) Presence of ϵ-N-Methyllysine (NML) in Proteus flagellins

AMINO ACID	148	149	150	151	152	153	154	155	10041	2815	128	166	232
Asp	51	59	59	60	57	52	59	54	64	55	62	63	56
Thre	25	33	27	32	33	28	32	33	29	34	30	26	33
Ser	26	20	25	24	21	25	21	26	24	26	28	24	28
Glu	45	46	50	47	51	46	49	48	48	49	39	45	43
Pro	2	2	2	2	4	5	3	3	2	2	2	2	2
Gly	31	26	30	24	26	30	25	27	28	26	29	30	24
Ala	45	42	47	43	45	49	44	40	46	42	41	49	44
Val	25	21	24	22	26	22	23	26	27	23	27	32	25
Met	5	4	5	5	5	4	5	5	5	5	6	5	6
Ileu	27	23	24	19	24	27	22	24	19	21	23	20	22
Leu	31	37	33	28	31	30	27	35	33	31	28	27	29
Tyr	6	6	6	3	4	6	6	4	4	3	6	3	3
Phe	10	10	7	11	12	10	7	10	10	9	9	12	12
His	2	0	0	0	0	0	3	1	0	0	0	2	1
Lys	21	21	17	16	19	20	20	17	19	17	31	24	31
NML	8	6	7	8	10	7	5	8	6	5	0	0	0
Arg	20	18	17	15	17	18	11	15	15	16	15	14	14
TOTAL	380	374	380	359	385	379	362	376	379	364	376	378	373

TABLE 4.1 Amino acid analysis of P. morganii flagellins

AMINO ACID	NCTC 4175	NCTC 10020	102
Asp	56	64	56
Thre	31	32	26
Ser	25	32	24
Glu	46	40	41
Pro	4	1	13
Gly	26	31	33
Ala	41	42	34
Val	33	30	27
Met	5	3	8
Ileu	24	24	20
Leu	28	32	30
Tyr	4	4	10
Phe	9	10	10
His	3	0	6
Lys	33	21	25
NML	0	0	E^-
Arg	14	14	16
TOTAL	382	380	379

TABLE 4•2 Amino acid analysis of P. vulgaris flagellins

E^- denotes the absence of NML residues as determined by pH 3.5 paper electrophoresis.

AMINO ACID	NCTC 6197	249	129
Asp	65	68	65
Thre	35	35	29
Ser	31	33	28
Glu	37	40	37
Pro	3	3	3
Gly	28	28	25
Ala	37	38	39
Val	27	27	25
Met	5	3	4
Ileu	23	21	26
Leu	32	31	31
Tyr	4	5	6
Phe	11	9	9
His	2	0	1
Lys	19	20	31
NML	0	0	E^-
Arg	15	14	15
TOTAL	374	375	374

TABLE 4·3 Amino acid analysis of P. mirabilis flagellins

E^- denotes the absence of NML residues as determined by pH 3.5 paper electrophoresis.

AMINO ACID	NCTC 6344	12	23
Asp	53	58	62
Thre	26	32	33
Ser	27	27	28
Glu	43	43	48
Pro	7	2	2
Gly	35	31	31
Ala	49	50	48
Val	29	29	33
Met	8	5	7
Ileu	19	25	21
Leu	27	29	28
Tyr	5	3	3
Phe	11	7	7
His	2	0	0
Lys	24	24	22
NML	0	E^-	E^-
Arg	16	16	15
TOTAL	381	381	388

TABLE 4·4 Amino acid analysis of P. inconstans flagellins

E^- denotes the absence of NML residues as determined by pH 3.5 paper electrophoresis.

AMINO ACID	NCTC 7459	9	7
Asp	54	52	47
Thre	31	31	28
Ser	24	28	28
Glu	42	44	36
Pro	5	2	12
Gly	26	28	38
Ala	39	43	35
Val	15	27	26
Met	13	3	9
Ileu	25	27	20
Leu	35	30	28
Tyr	3	2	11
Phe	8	11	16
His	6	0	3
Lys	25	26	26
NML	0	E^-	E^-
Arg	19	15	16
TOTAL	370	369	379

TABLE 4.5 Amino acid analysis of P. rettgerri flagellins

E^- denotes the absence of NML residues as determined by pH 3.5 paper electrophoresis.

(i) Quantitative analysis

NML was resolved from lysine using an extended amino acid analysis procedure. Analysis of a standard mixture of amino acids to which authentic NML had been added showed that NML was eluted immediately after lysine, which is eluted after histidine (Figure 1·3). Quantitative analysis of 13 P. morganii flagellins, and of P. vulgaris NCTC 6197 and 249; P. inconstans NCTC 6344 and P. rettgerri NCTC 7475 flagellins revealed that the presence of NML is restricted to certain P. morganii strains (Figure 4·1). The latter could be divided into three groups with NML/lysine ratios of approximately 0.3, 0.4 or 0.5 (Table 4·6). Salmonella g.... antigenic flagellins have a NML/lysine ratio of approximately 0.8 (Section 1).

Flagellins of P. morganii strains 166, 128 and NCTC 232 did not possess any NML residues. However, their lysine content was increased relative to the flagellins of the other P. morganii strains.

(ii) Qualitative analysis

Hydrolysates of the P. mirabilis, P. rettgerri, P. vulgaris and P. inconstans flagellins which had not been analysed quantitatively for the presence of NML, were subjected to pH 3.5 paper electrophoresis in the presence of suitable markers. NML appears as a well-resolved band between those of lysine and arginine on the electrophoretogram. No NML was detected in the Proteus flagellins examined by this method, which is sensitive to 1 NML residue; however, P. morganii flagellins shown to contain NML by amino acid analysis, gave a positive result.

c) Polyacrylamide gel electrophoresis

(i) Homogeneity and mobility of Proteus flagellins

When Proteus flagellins were subjected to pH 2.7 polyacrylamide gel electrophoresis in 8M urea, a single band was obtained in each case (Figure 4·2).

A comparison of the mobilities of flagellins of the P. morganii

FIGURE 4.1

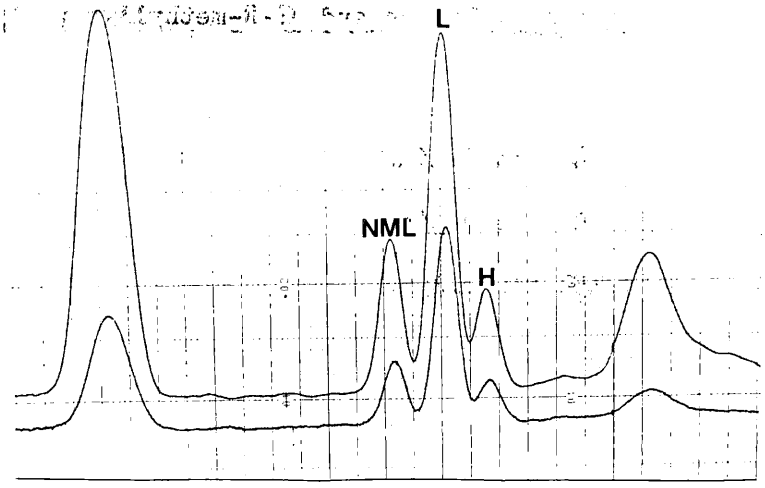
Separation of histidine, lysine and ∈-N-methyllysine by automatic amino acid analysis.

a) P. morganii 148 flagellin.

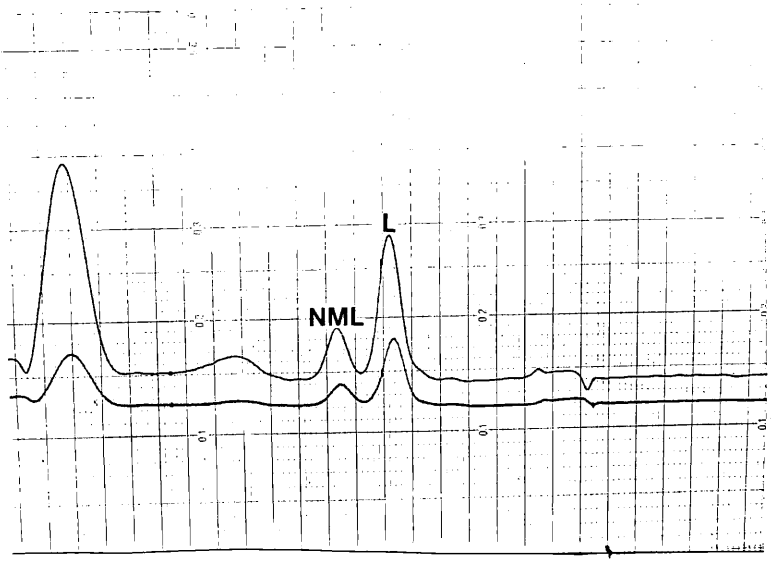
b) P. morganii 149 flagellin.

c) P. morganii 151 flagellin.

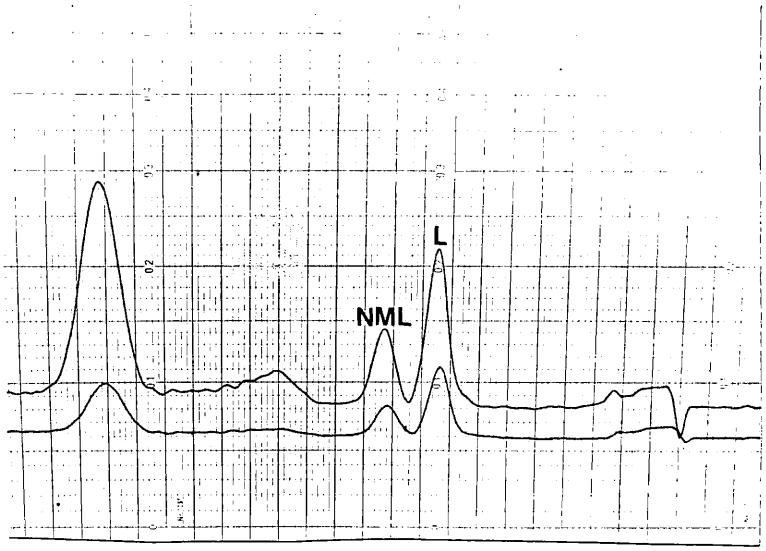
a



b



c



P. morganii strain	NML/Lys RATIO
149 154 NCTC 10041 NCTC 2815	0.3
148 150 153	0.4
151 152 155	0.5
166 NCTC 232 128	0

TABLE 4.6 ϵ-N-Methyllysine/lysine ratios of P. morganii flagellins

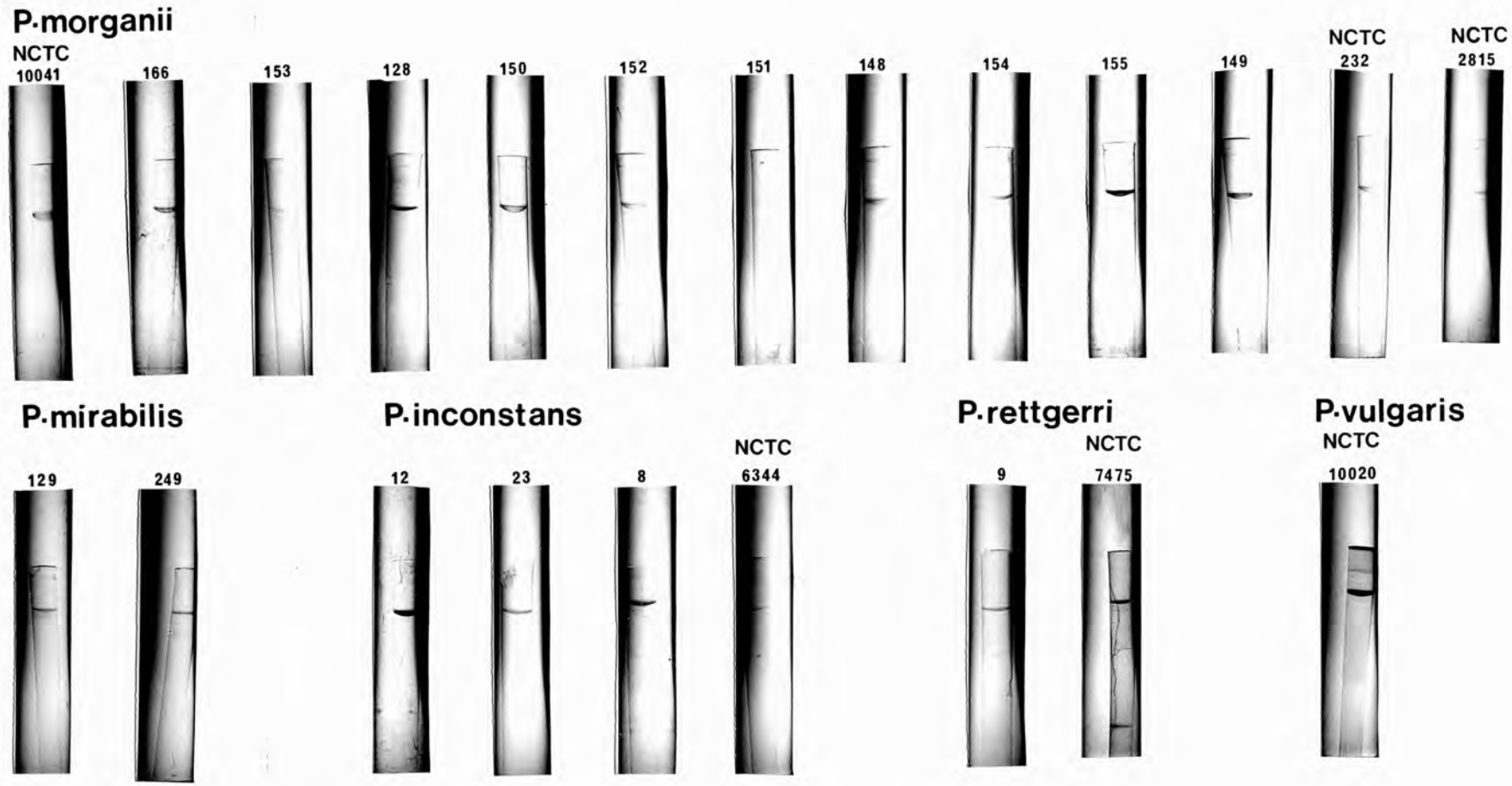


FIGURE 4·2 pH 2.7 polyacrylamide gel electrophoresis of Proteus flagellins in 8M urea.

strains with those of the P. rettgerri, P. vulgaris, P. mirabilis and P. inconstans strains on polyacrylamide gels at pH 2.7 in the presence of 8M urea revealed very little difference in mobility both within and between flagellins isolated from different Proteus species. It is possible that electrophoresis of mixtures of flagellins on single gels, in conjunction with longer running times and softer gels (lower acrylamide concentration), may have allowed differentiation of the flagellins.

(ii) Cyanogen bromide digests of Proteus flagellins: Estimation of molecular weights of CNBr fragments.

Cyanogen bromide digests of P. vulgaris NCTC 10020, P. morganii NCTC 2815 and P. mirabilis NCTC 6197 flagellins were prepared. The flagellins have 5, 3 and 5 methionine residues, respectively. The expected number of CNBr fragments would, therefore, be 6, 4 and 6, respectively.

The CNBr digests were run on pH 2.7 polyacrylamide gels in 8M urea; and on SDS polyacrylamide gels at pH 8.8 (Figure 4.3) so that molecular weight estimations of the CNBr fragments could be made (Table 4.7).

A band corresponding to the reported molecular weight of Proteus flagellins was observed in the P. morganii CNBr digest subjected to SDS polyacrylamide gel electrophoresis.

In all three CNBr digests, there were 2 faint, high molecular weight bands which were assumed to correspond to small amounts of partially degraded flagellin. In addition, there were 2 strong bands corresponding to molecular weights of about 21,000 and 11,000. In the P. vulgaris CNBr digest, the higher molecular weight fragment formed a double band. In the P. morganii CNBr digest, 2 additional fainter bands of intermediate molecular weight were also present.

In all three CNBr digests, a third diffuse band was present at the bottom of the gel; it was not possible to determine its molecular

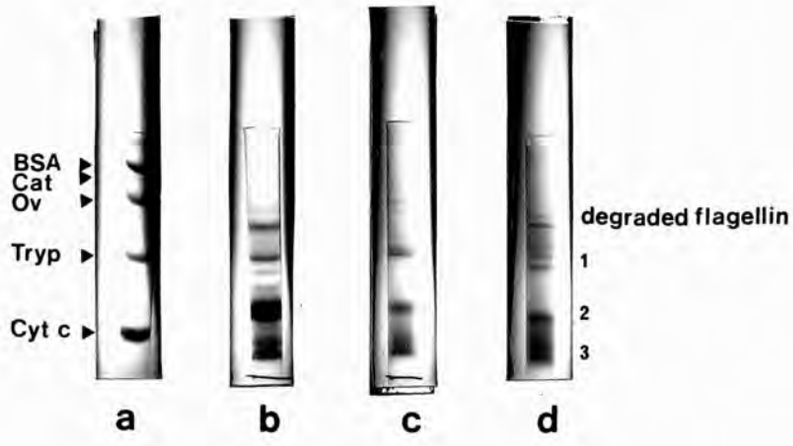


FIGURE 4.3

SDS polyacrylamide gel electrophoresis at pH 8.8 of CNBr digests of Proteus flagellins (15% gels).

a) Mixture of marker proteins

b) P. morganii NCTC 2815

c) P. mirabilis NCTC 6197

d) P. vulgaris NCTC 10020

BAND	P. mirabilis NCTC 6197	P. morganii NCTC 2815	P. vulgaris NCTC 10020
Flagellin		41,210	
Partially degraded flagellin	38,020 35,320	37,580 31,260	37,580 34,670
1	21,380	21,380 17,950 15,480	23,170 21,380
2	11,220	11,480	11,350
3	ND	ND	ND

TABLE 4.7 Molecular weight determination of CNBr fragments of Proteus flagellins by SDS polyacrylamide gel electrophoresis, pH 8.8 on 15% gels

ND - Not determined

weight. This band probably represents the smaller CNBr fragment(s) that were unresolved on the gels.

d) Fractionation of CNBr digests of *Proteus* flagellins

Some preliminary work was done on the fractionation of CNBr digests of *P. vulgaris* NCTC 10020, *P. morganii* NCTC 2815 and *P. mirabilis* NCTC 6197 flagellins by gel filtration on Sephadex columns. In each case, the freezedried CNBr digest was fully soluble in 98-100% formic acid. The fractions were monitored by polyacrylamide gel electrophoresis at pH 2.7 in 8M urea, pooled and freezedried.

When the CNBr digest of *P. morganii* CNTC 2815 flagellin was fractionated, the fastest moving fragment on the polyacrylamide gels was eluted from the Sephadex column before the next fastest moving band. When the CNBr digests of the other *Proteus* flagellins were fractionated, the fragments were eluted from the column in the same order as they appeared on the gels, that is, the slowest moving was eluted first and the fastest moving eluted last.

5. AMINO ACID COMPOSITIONS OF VARIOUS BACTERIAL FLAGELLINS

a) Polyacrylamide gel electrophoresis

The flagellins were subjected to pH 2.7 polyacrylamide gel electrophoresis in 8M urea to determine their homogeneity. In each case, a single band was observed.

b) Amino acid analysis

The amino acid compositions of various bacterial flagellins are given in Table 5·1. All the flagellins had a high proportion of aspartic and glutamic acids (or amides) and alanine, and little proline, methionine, tyrosine, phenylalanine or histidine. Methionine and histidine were absent from Alcaligenes faecalis flagellin; histidine was also undetected in Erwinia carotovora flagellin. Cysteine or cysteic acid were not present in any of the flagellins analysed.

E. coli K12 flagellin differed from the other flagellins analysed in that it had a much greater tyrosine/phenylalanine ratio, and a lower arginine content. Erwinia carotovora and Alcaligenes faecalis flagellins had lower proportions of lysine residues than the other flagellins.

Only one tyrosine residue was detected in the Bacillus, E. carotovora and R. rubrum flagellins, and two in Ps. aeruginosa flagellin. These findings are of particular interest when the rôle of tyrosine residues in the structural and functional requirements of the bacterial flagella are considered.

ϵ-N-methyllysine residues were absent from all the flagellins analysed, including E. coli 8666 flagellin, as determined by pH 3.5 paper electrophoresis.

AMINO ACID	B. subtilis 168	B. sphaericus NCIB 9370	B. pumilis NCIB 9369	E. coli K12	E. carotovora
Asp	57	57	58	67	62
Thre	22	35	22	46	42
Ser	28	29	21	32	44
Glu	48	41	49	32	35
Pro	3	1	4	5	4
Gly	22	25	33	37	30
Ala	48	46	47	50	54
Val	15	13	17	26	18
Met	9	11	9	3	8
Ileu	26	22	27	21	29
Leu	35	29	36	28	33
Tyr	1	1	1	8	1
Phe	5	7	8	4	4
His	4	7	6	1	0
Lys	21	20	21	21	11
NML	ND	E^-	E^-	E^-	E^-
Arg	16	17	17	9	20
TOTAL	360	361	376	390	395

TABLE 5·1 Amino acid compositions of various bacterial flagellins

E^- Absence of NML as determined by pH 3.5 paper electrophoresis.

ND - Not determined.

AMINO ACID	S. marcescens NCIB 9155	Ps. fluorescens NCTC 10038	Ps. aeruginosa NCIB 8295	R. rubrum NCIB 8255	Alc. faecalis NCIB 8156
Asp	60	45	56	56	64
Thre	29	38	27	31	25
Ser	30	36	29	26	34
Glu	30	33	42	49	43
Pro	11	10	5	5	8
Gly	34	36	32	31	36
Ala	40	62	50	47	62
Val	32	25	25	14	25
Met	4	6	12	11	0
Ileu	20	20	24	19	26
Leu	31	24	29	24	27
Tyr	8	7	2	1	4
Phe	12	10	6	6	10
His	2	2	5	4	0
Lys	28	16	20	18	10
NML	E^-	E^-	E^-	E^-	E^-
Arg	15	15	18	19	17
TOTAL	386	385	382	361	391

TABLE 5.1 contd.

6. CHEMICAL MODIFICATION OF TYROSINE AND ARGININE RESIDUES IN BACTERIAL FLAGELLINS

a) Arginine residues

1,2 - cyclohexanedione was used for the selective modification of arginine residues in *B. subtilis* 168, *B. sphaericus* NCIB 9370, *Erwinia carotovora*, *P. morganii* 152, *E. coli* K12, *S. paratyphi* B, (SL 174) H1-*b* and *S. senftenberg* (SL 736), H1-*g,s,t* flagellins.

The extent of the reaction was determined by amino acid analysis of the modified samples. (Values were calculated for a flagellin molecular weight of 40,000.) In Table 6·1, the results of the amino acid analysis of modified *S. senftenberg* SL 736, H1-*g,s,t* flagellin (monomer) is presented. This can be compared to the analysis of native flagellin treated identically only without 1,2 - cyclohexanedione addition, serving as a control sample. No other amino acids, besides the arginine residues, are affected significantly by the 1,2 - cyclohexanedione, at least as far as can be detected by amino acid analysis. The number of modified arginine residues was calculated both from the diminution of the arginine peak in the amino acid analysis relative to the control sample, and directly from the elution peak of modified arginine which appears just after phenylalanine. The single product of modification with 1,2 - cyclohexanedione is, in fact, N^7,N^8 - (1,2 - dihydroxycyclohex - 1,2 - ylene) - L - arginine (DHCH - arginine). However, upon hydrolysis in 6N HCl at 110°C for 24 hours, this product is destroyed, resulting in an 18-20% regeneration of arginine. If hydrolysis is carried out in the presence of excess mercaptoacetic acid (20µl), on the other hand, DHCH - arginine is converted to a neutral product which elutes just after phenylalanine. This neutral product is referred to as "modified arginine". The numbers of modified and unmodified arginine residues present after treatment of the monomeric and polymeric forms of various bacterial flagellins with 1,2 -

AMINO ACID	NATIVE FLAGELLIN	MODIFIED FLAGELLIN
Asp	68	67
Thre	35	35
Ser	34	35
Glu	37	36
Pro	3	3
Gly	31	31
Ala	48	47
Val	28	28
Met	3	3
Ileu	23	22
Leu	28	29
Tyr	7	8
Phe	10	10
MODIFIED Arg	0	5
His	0	0
Lys	26	29
Arg	12	1

TABLE 6·1 Amino acid analyses of native and 1,2 - cyclohexanedione-treated S. senftenberg, SL 736, flagellin.

cyclohexanedione are given in Table 6·2. Also shown are the total number of arginine residues present in the flagellins before modification. When the monomeric form of each flagellin is modified, about half or more of the arginine residues are not recovered either in the modified or unmodified state. This is also true when the polymeric form of S. senftenberg SL 736, S. paratyphi B SL 174, B. subtilis 168 and E. coli K12 flagellins are modified. However, about three quarters of the arginine residues are recovered when the polymeric form of the other bacterial flagellins are treated with 1,2 - cyclohexanedione.

In addition, more arginine residues are modified when the flagellins are treated in the monomeric rather than in the polymeric form as shown by the greater number of modified arginines and smaller number of unmodified arginines present. Thus, it was assumed that the arginine residues not detected by amino acid analysis were probably modified, but were susceptible to degradation under the conditions of hydrolysis.

To try to improve the recovery yield of modified arginine residues, the conditions under which hydrolysis of modified S. senftenberg SL 736 flagellin monomer was carried out were varied (Table 6·3). In one experiment, a greater excess of mercaptoacetic acid (60 instead of 20µl) was included during hydrolysis on the assumption that DHCH - arginine would then be converted to the neutral product more efficiently. However, this resulted in a loss, rather than an increase, in the number of modified arginines detected.

When hydrolysis is carried out in the absence of mercaptoacetic acid, DHCH - arginine is destroyed. There is an 18 to 20% regeneration of arginine; the remainder is converted to unknown basic products that are not eluted from the amino acid analyser under normal conditions. These results were confirmed; the 5 modified arginine residues,

	MODIFICATION			
	BEFORE	AFTER		
FLAGELLIN	TOTAL ARG	ARG	MODIFIED ARG	TOTAL ARG	FORM
NATIVE *S. senftenberg* SL 736	12	0	6	6	MON
		7	2	9	POL
S. senftenberg SL 736	12	1	5	6	MON
		3	3	6	POL
S. paratyphi B SL 174	12	1	5	6	MON
		2	4	6	POL
B. sphaericus NCIB 9370	17	2	3	5	MON
		13	1	14	POL
B. subtilis 168	15	3	2	5	MON
		7	0	7	POL
Erwinia carotovora	20	2	5	7	MON
		13	3	16	POL
Proteus morganii 152	17	3	4	7	MON
		11	2	13	POL
E. coli K12	9	1	4	5	MON
		2	3	5	POL

TABLE 6·2 Arginine content of the monomeric (MON) and polymeric (POL) forms of various bacterial flagellins, before and after modification with 1,2 - cyclohexanedione

HYDROLYSIS CONDITIONS	ARG	MODIFIED ARG	TOTAL ARG
6N HCl 16 hrs + 20µl M	1	5	6
6N HCl 16 hrs + 60µl M	1	3.5	4.5
6N HCl 16 hrs - M	2	0	2
6N HCl 1 hr + 20µl M	1	Several peaks	-
2N HCl 16 hrs + 20µl M	Incomplete hydrolysis		

TABLE 6·3 Effect of different hydrolysis conditions on the recovery of modified arginine residues of S. senftenberg SL 736 flagellin (monomer) treated with 1,2 - cyclohexanedione

M = mercaptoacetic acid

detectable by amino acid analysis when 20µl mercaptoacetic acid are included during hydrolysis, were no longer present, but one additional unmodified arginine was recovered.

Reduction of the hydrolysis incubation time from 16 hours to 1 hour resulted in the replacement of the single, modified arginine peak by several, poorly resolved peaks; the resolution of the other amino acid peaks were unaffected.

Incubation with 2N HCl instead of 6N HCl resulted in incomplete hydrolysis.

Table 6·2 also shows the results obtained when native S. senftenberg SL 736 flagellin was modified. Native flagellin (monomer) was obtained by acid dissociation of a flagella pellet (polymer). In the case of the other flagellins, the flagella pellet had first been acid dissociated and then repolymerised by precipitation with ammonium sulphate to give a purified polymer. Monomeric flagellin was obtained by acid dissociation of the purified polymer.

7 arginine residues remainded unmodified in the native Salmonella polymer as compared to 3 in the purified polymer. On the other hand, the native monomer contained no unmodified arginine residues; only one was detected in the purified monomer flagellin.

b) Tyrosine residues

Tetranitromethane was used for the selective modification of tyrosine residues in S. senftenberg SL 736, H1-g,s,t; S. paratyphi B SL 174, H1-b and E. coli K12 flagellins.

The extent of the reaction was determined by amino acid analysis of the modified samples. (Values were calculated for a flagellin molecular weight of 40,000.) A comparison of the amino acid analysis of modified S. senftenberg SL 736 flagellin and that of native flagellin, presented in Table 6·4, shows that no other amino acids except for the tyrosines are affected significantly by tetranitromethane,

AMINO ACID	FLAGELLIN	MODIFIED FLAGELLIN
Asp	57	59
Thre	44	43
Ser	28	29
Glu	29	29
Pro	0	0
Gly	29	29
Ala	48	48
Val	31	31
Met	3	3
Ileu	17	17
Leu	29	28
Tyr	9	7
Phe	7	7
His	1	1
Lys	25	24
Arg	12	11

TABLE 6.4 Amino acid analysis of native and tetranitromethane-treated S. senftenberg SL 174 flagellin

as shown by amino acid analysis. The number of modified tyrosine residues was calculated from the decrease in the tyrosine peak in the amino acid analysis relative to the control sample, which was treated identically only without the addition of tetranitromethane (TNM).

The product of modification with TNM, 3 - nitrotyrosine, elutes from the column immediately after phenylalanine. However, although authentic 3-nitrotyrosine was located in this position, no corresponding peaks were found in the modified samples. Unidentified peaks, however, were present in both modified samples and their controls.

In Table 6·5 are shown the numbers of tyrosine residues present in the three flagellins before and after treatment with TNM. The monomeric flagellin and polymeric flagellum were modified in each case. No tyrosine residues were nitrated in E. coli K12 flagellin monomer or polymer. In the Salmonella flagellins, more tyrosine residues were nitrated in the monomeric than in the polymeric flagellin.

FLAGELLIN	FORM	MODIFICATION BEFORE	MODIFICATION AFTER	TYROSINE RESIDUES
S. senftenberg SL 736	MONOMER	7	6	
	POLYMER		7	
E. coli K12	MONOMER	8	8	
	POLYMER		8	
S. paratyphi B SL 174	MONOMER	9	6	
	POLYMER		7	

TABLE 6·5 Tyrosine content of monomeric and polymeric forms of bacterial flagellins before and after modification with tetranitromethane.

DISCUSSION

The object of this research was to gain a more detailed chemical knowledge of bacterial flagellins. Such information would be useful in determining the detailed basis of H antigenic variation and the location of antigenic sites; the sites of amino acid substitutions ~~responsible for~~ resulting from mutations affecting flagella shape and function; the presence of ε-N-methyllysine residues in certain flagellins; possible conformational changes which occur in the assembly of flagellin subunits into flagellar filaments and phylogenetic relationships between bacteria.

Antigenic variation is particularly well characterised in the genus Salmonella, although a less extensive analysis has also been performed for other genera, for example, Escherichia and Proteus.

H antigenic differences in Salmonella are related to differences in amino acid composition of the flagellin molecules, the variation being considerable for non-cross-reacting antigens (McDonough, 1965). As most Salmonella species are diphasic with the potential to make flagella of two antigenic types, the monophasic Salmonella g.... antigenic strains, which possess only one structural gene and therefore make flagellin of only one antigenic type, were particularly suitable for the work to be carried out. A large number of sub-component variants of g.... antigens are well characterised (g,s,t; g,m; g,p; f,g etc.), presumably due to small changes in amino acid sequence of these flagellins.

Studies by Parish and Ada (1969) have shown that Salmonella flagellin of antigenic type f,g is cleaved by cyanogen bromide to give four fragments which are readily purified for further analysis. On the basis of polyacrylamide gel electrophoresis analysis, amino acid analysis and carboxypeptidase treatment of flagellin and fragments,

they proposed a sequence for the fragments which was later confirmed by Davidson (1971):

N-terminal C-terminal

B ——— A —— D ——— C

Flagellin of antigenic type g,s,t was chosen for the present study as Salmonella strains carrying these antigens gave excellent flagella yields (400-700mg/20 trays) when cultured on enriched agar medium. Salmonella strains carrying the f,g antigens, on the other hand, gave very poor yields (50-100mg/20 trays).

The cleavage of S. senftenberg (SL 736) g,s,t flagellin with cyanogen bromide (CNBr) gave very comparable results with those obtained for S. adelaide f,g flagellin by Parish and Ada (1969). In both cases, 70% formic acid was found to be a suitable solvent for CNBr digestion, probably because it is a strong dissociating agent and also maintains a low pH. CNBr digests of the Salmonella g.... antigenic flagellins g,s,t; g,p; g,m and f,g each showed an identical pattern of bands to that of S. adelaide flagellin on pH 2.7 polyacrylamide gels in the presence of 8M urea. Although CNBr fragments A, B and D were resolved by gel filtration on Sephadex columns, it was not possible to obtain pure CNBr C fragment in any quantity by these techniques. However, it was possible to obtain reasonable quantities of 'C,D' complex by exposing the flagellin to the oxidant chloramine-T before treatment with CNBr. Presumably chloramine-T oxidises the more exposed methionine residues to methionine sulphoxide, which is not susceptible to CNBr cleavage. Thus the methionine residue linking CNBr C and CNBr D must be very accessible to chloramine-T, whereas the methionine residue linking 'C,D' complex to the rest of the flagellin molecule is probably masked and therefore less readily oxidised. The 'C,D' complex was invariably contaminated with small amounts of CNBr A and CNBr B suggesting that the methionine residue linking these two fragments must

be at least partially inaccessible to chloramine-T. Parish and Ada, however, were able to obtain 'C,D' complex of S. adelaide flagellin in a pure form using the same experimental procedure.

The amino acid compositions of nine Salmonella g.... antigenic flagellins were very similar and showed good agreement with those analysed by McDonough (1965). However, there was one exception, that of g,q flagellin whose amino acid composition was more similar to other Salmonella antigens such as l,2; a; b; r; and e,n,x (McDonough, 1965) than to the other g.... antigenic flagellins. The most important difference was the possible presence of histidine in g,q flagellin which was not detected in any of the other g.... antigenic flagellins analysed. Comparison of the amino acid composition of SL 175 f,g flagellin and that of S. adelaide f,g flagellin (Parish and Ada, 1969) showed some differences; aspartic acid content was higher, and glycine and alanine contents lower in the latter flagellin. A more striking difference was the absence in the former, and presence in the latter of ϵ-N-methyllysine (NML) residues. Stocker, McDonough and Ambler (1961) located a gene close to, but separable from the H1 structural gene for flagellin, which determined the presence or absence of NML in flagellins. Approximately equimolar amounts of lysine and NML residues have been reported in Salmonella flagellins (McDonough, 1965; Parish and Ada, 1969), a NML/lysine ratio of about 0.3:1 in certain P. morganii flagellins (Barr, 1973), and of about 0.12:1 in S. serpens flagellin (Martinez, 1963b). Methylated lysine residues have also been detected in histones (Paik and Kim, 1967) and several cytochromes (DeLange, Glazer and Smith, 1969).

NML/lysine ratios for the Salmonella g.... antigenic flagellins were somewhat lower than those reported by McDonough (1965); approximately 0.8 compared to 1.0. NML residues were detected, either by quantitative (automatic amino acid analysis) or qualitative (pH 3.5

paper electrophoresis) techniques, in all the g.... antigenic flagellins analysed except those of the g,q and f,g antigenic types. In these two latter flagellins, the number of lysine residues was approximately equal to the sum of lysine residues plus NML residues in the other g.... antigenic flagellins.

The NML/lysine ratios for P. morganii flagellins were lower than those observed for the Salmonella g.... antigenic flagellins, and could be differentiated into three groups with NML/lysine ratios of approximately 0.3, 0.4 or 0.5. NML residues were absent from flagellins of P. morganii strains 166, 128 and NCTC 232 and all the P. mirabilis, P. vulgaris, P. inconstans and P. rettgerri strains analysed. Flagellins isolated from bacteria of various other genera were also shown to contain no NML residues.

No correlation between the presence or absence of NML, and any other amino acid content was observed. It is of note, however, that the mole % guanine plus cytosine content of both Salmonella and P. morganii strains is 50-52, as compared to 38-40 for the other Proteus strains (Marmur, Falkow and Mandel, 1963). It would be expected that the DNA's extracted from closely related species would have similar base composition, whereas those of widely different species are likely to have widely different base composition. However, a comparison of the amino acid compositions of P. morganii flagellins with those of the other Proteus species reveals very little difference in the relative proportions of amino acids. Only the numbers of proline and methionine residues showed any significant variation; they were low in the P. morganii and Salmonella g.... antigenic flagellins, but higher and more variable among the other Proteus flagellins.

Comparison of the amino acid compositions of the g,s,t CNBr fragments and those of S. adelaide f,g flagellin (Parish and Ada, 1969) reveals more variation between their CNBr A peptides than between their

other CNBr fragments. Parish and Ada suggested that in the polymer the fragment A region is exposed; antigenic differences would, therefore, be expected to be located in that part of the molecule, and manifested as amino acid differences.

The amino acid composition of CNBr A is of particular interest as, although it contains approximately half of the total lysine, it contains 9 of the 11 NML residues of the molecule. As fragment A appears to be exposed in the polymer, and methylation of lysine is enzymically mediated (Kim and Paik, 1965), Parish and Ada suggested that methylation of the flagellin lysine residues occurred only after polymerisation of the flagellin. In addition, fragment A contains only one of the 12 arginine residues of the molecule. Benoiton and Deneault (1966) reported that peptide bonds of a protein incorporating the COOH groups of NML will be hydrolysed by trypsin at a slower rate than those incorporating COOH groups of lysine. Thus, fragment A would be expected to be relatively resistant to trypsin because it contains only one arginine and several NML residues. However, the distribution of these residues will also affect how efficiently the fragment is digested. Tryptic digests of CNBr A fragment of g,s,t flagellin run on pH 2.7 polyacrylamide gels in the presence of 8M urea consistently revealed two bands with similar electrophoretic mobility, suggesting that a region of the fragment remains undigested by trypsin.

The N-terminal amino acid of the nine Salmonella g.... antigenic flagellins analysed was tentatively identified as alanine. Alanine has also been identified as the N-terminal amino acid of flagellin in P. vulgaris (Kobayashi, Rinker and Koffler, 1959), P. mirabilis (Glossman and Bode, 1972) and other Proteus strains (Barr, 1973), S. adelaide (Parish and Ada, 1969), S. typhimurium (Joys and Rankis, 1972) and S. marcescens (Kobayashi, Rinker and Koffler, 1959).

Close homology has been demonstrated beyond the N-terminal residue

between P. vulgaris NCTC 10020 and P. rettgerri NCTC 7475 (Barr, 1973) and Salmonella H2 - e,n,x (Simon, 1980) flagellins:

Salmonella

H2 - e,n,x N - Ala - Gln - Val - Ileu -

P. vulgaris

NCTC 10020 N - Ala - Leu - Val - Gly -

P. rettgerri

NCTC 7475 N - Ala - Leu - Val - Gly -

Residues identical in two flagellins are underlined.
Double underlining indicates identities in three flagellins.

The N-terminal amino acid of both CNBr A and CNBr B fragments of g,s,t flagellin was identified as alanine. Although the N-terminal amino acid of CNBr B fragment of S. adelaide f,g flagellin was also identified as alanine, that of CNBr A was reported as lysine (Davidson, 1971). This may represent a genuine amino acid difference between the two antigenic types, as the alanine content of the g,s,t CNBr A fragment was significantly greater than that of the f,g CNBr A fragment. As both the N-terminal amino acid of S. adelaide f,g flagellin and of its fragment B, but not of its fragment A, is alanine, Parish and Ada concluded that CNBr B fragment was the N-terminal peptide. This assumption was also made for g,s,t flagellin although the N-terminal analysis was not able to confirm this.

The N-terminal amino acid of CNBr D of g,s,t flagellin was identified as phenylalanine, in agreement with Davidson's results.

C-terminal analysis of Salmonella g.... antigenic flagellins indicated a common C-terminal amino acid sequence:

..... Lys - Ser - Leu - Leu - ArgCOOH

The latter two amino acids also form the C-terminal sequence of B. subtilis 168 and W23 (DeLange et al., 1976), P. mirabilis (Glossman and Bode, 1972) and S. adelaide (Davidson, 1971) flagellins; close

homology may well extend considerably beyond these residues. B. subtilis 168 and W23 flagellins differ totally in antigenic character (Martinez, Brown and Glazer, 1967) and significantly in amino acid composition (DeLange et al., 1973). However, comparison of the sequences of the amino- and carboxyl-terminal CNBr peptides derived from the two proteins, demonstrated only 3 conservative substitutions (for example, glycine replaced by serine), all in the amino-terminal peptide. The carboxyl-terminal CNBr peptides were identical. Thus it seems likely that the N- and C-terminal portions of the flagellin molecule have conserved amino acid sequences essential for the structural and/or functional requirements of the molecule. The CNBr fragments A, B and D of g,s,t flagellin were also subjected to C-terminal analysis. Homoserine should have been the first amino acid released in each case as methionine, the C-terminal amino acid of each fragment, is converted to homoserine by reaction with CNBr. However, homoserine was not easily resolved by the system used. The second amino acid released from the C-terminal of CNBr A was threonine. This agrees with the results obtained for CNBr fragment A of S. adelaide flagellin (Davidson, 1971). However arginine and leucine were released from both CNBr B and D indicating that the samples were probably contaminated with flagellin and/or CNBr C which has been confirmed as the C-terminal CNBr peptide in S. adelaide flagellin (Davidson, 1971).

Molecular weight estimations of Salmonella g.... antigenic flagellins by SDS polyacrylamide gel electrophoresis (PAGE) were in good agreement with those reported by McDonough and Smith (1967); a value of approximately 56,000 was obtained. However, previous investigations using the approach-to-equilibrium method or from sedimentation and diffusion data have suggested a value of 40,000 for Salmonella flagellins (McDonough, 1965). This discrepancy was not observed with P. mirabilis flagellin which gave a consistent value of around 40,000

using sedimentation equilibrium (Chang, Brown and Glazer, 1969) or SDS PAGE (Glossman and Bode, 1972). Similarly, Mirsky (1970) reported a molecular weight of approximately 34,000 for B. megaterium KM using either sedimentation equilibrium measurements or SDS PAGE.

It is assumed that mobility of a protein in SDS PAGE is governed only by its molecular weight (Shapiro, Vinuela and Maizel, 1967; Weber and Osborn, 1969) except for glycoproteins and very basic proteins, for example, histones (Hayashi, Matsutera and Ohba, 1974) which have been shown to behave aberrantly. However, it has been observed that apparent molecular weight values calculated from SDS PAGE do not always agree with those calculated from sequence data, for example, in B. subtilis 168 flagellin (Simon et al., 1977). In a study of several B. subtilis flagellins, these workers observed that mobility of certain of these proteins on SDS gels depended upon details of sample pretreatment. The anomalous mobilities observed suggested that there were residual interactions within the flagellin molecules in the presence of SDS, and, consequently, these proteins did not behave as ideal random coils. Similar effects have been reported in a comparison of E. coli and Salmonella flagellins (Kondoh and Hotani, 1974). Evidence that a single amino acid substitution could cause a shift in the apparent molecular weight as estimated by SDS PAGE is just beginning to emerge. De Jong et al. (1978) have shown that single amino acid differences in the protein α-crystallin (A chain) can result in SDS PAGE mobility changes. In their study, neutral substitutions of hydrophobic residues for hydrophilic ones increased electrophoretic mobility. Noel, Nikaido and Ames (1979), in a study of the histidine transport protein in S. typhimurium, reported that the substitution of cysteine (neutral) for arginine (cationic) at a site in the interior of the polypeptide chain caused the protein to migrate more slowly than the wild-type on SDS gels, as if its molecular weight were greater by as much as 2,000.

This was found to be a local SDS-polypeptide effect, due not only to the individual arginine (cysteine) residue, but also to the surrounding residues. This effect may be due to increased SDS binding or to a difference in conformation of SDS-protein complexes.

An increase in the number of methyl groups attached to the methyl-accepting chemotaxis proteins (MCP) in E. coli has also been shown to cause an increase in mobility on SDS gels (Boyd and Simon, 1980). This was explained in terms of increased SDS binding.

In the case of Salmonella g.... antigenic flagellins, the difference between the molecular weight values obtained by SDS PAGE and by analytical ultracentrifugation methods is particularly striking. This phenomenon has not been observed for Proteus flagellins. The molecular weights of bacterial flagellins, as determined by SDS PAGE, cover a wide range. In some genera, for example Proteus and Salmonella, the molecular weights differ only by a few thousand; in others, for example Bacillus and Escherichia, the differences are considerable (McDonough and Smith, 1976). Based on a knowledge of their amino acid sequences, the behaviour of bacterial flagellins on SDS gels would therefore provide an ideal system for the study of the amomalous electrophoretic behaviour of proteins in the presence of SDS.

The molecular weight (27,000) of the CNBr A fragment of g,s,t flagellin, as determined by SDS PAGE, correlates with that of the whole flagellin estimated by the same method. However its molecular weight based on an arginine content of one residue was estimated as 19,000, in agreement with Parish and Ada's results for the CNBr A fragment of S. adelaide f,g flagellin. The presence of two arginine residues is unlikely as this would give a molecular weight value of 38,000, exceeding even the SDS PAGE value. However, the use of a stain specific for arginine residues on a tryptic peptide map of g,s,t CNBr A was not able to clarify the point as rather inconclusive results were

obtained. This was also true when the lysine residues of g,s,t CNBr A were modified by trifluoracetylation or citraconylation in order to limit trypsin digestion to the single arginine residue. This could have been due to the location of the arginine residue as, if it was positioned at one end of the fragment, cleavage would have resulted in the formation of one large and one very small peptide.

In contrast, the molecular weight of the CNBr B fragment of g,s,t flagellin was estimated as approximately 12,000 using either SDS PAGE or amino acid analysis. Thus it was concluded that, if the flagellin molecule is behaving anomalously on SDS gels, then this might be due to the conformation of the CNBr A region in the molecule. CNBr A contains just under half of the hydrophobic and of the basic residues present in the whole flagellin molecule. The hydrophobic residues make up 36% of the amino acid content of the CNBr A fragment. When hydrophobic residues were substituted for hydrophilic ones in the α-crystallin (A chain) protein, an increase in electrophoretic mobility on SDS gels resulted (De Jong et al., 1978). The low mobility of collagen peptides on SDS PAGE was accounted for by their low content of hydrophobic residues (Hayashi and Nagai, 1980). Thus, it seems likely that the hydrophobic nature of a protein is responsible for its anomalous behaviour on SDS gels, although whether this results in a lower or higher molecular weight value than expected will probably depend upon the conformation of each individual protein.

Another interesting feature of the amino acid composition of the g,s,t CNBr A fragment is that it contains 9 of the 11 NML residues of the molecule. Boyd and Simon (1980) reported that the more methyl groups bound to MCP in E. coli, the faster it ran electrophoretically. In contrast, CNBr A was electrophoretically slower than would be expected.

For ease of comparison with other workers' amino acid analysis

results, all amino acid values were calculated for a flagellin molecular weight of 40,000. Further investigations are necessary to confirm which molecular weight value is the most valid.

The molecular weights of the CNBr fragments of P. mirabilis flagellin, as determined by SDS PAGE, were in good agreement with those reported by Glossman and Bode (1972). Fragments of corresponding molecular weight were also present in P. vulgaris and P. morganii CNBr digests. One of the fragments formed a double band in the P. vulgaris CNBr digest; additional peptides of intermediate size were observed in the P. morganii CNBr digest. The smaller CNBr fragments were unresolved at the bottom of the gel, thus preventing molecular weight determination.

Based on knowledge of molecular weights and of the end groups of flagellin and the CNBr fragments, Glossman and Bode (1972) established the CNBr peptide sequence of P. mirabilis flagellin and compared it to that of S. adelaide flagellin (Parish and Ada, 1969; Davidson, 1971). They concluded that the largest P. mirabilis CNBr fragment (P2) probably corresponded to CNBr fragments A and D of S. adelaide; the methionine residue linking A and D in S. adelaide is missing in P. mirabilis flagellin.

Investigations have started on the fractionation of CNBr digests of Proteus flagellins to determine the extent of homology between Proteus and Salmonella flagellins. Polyacrylamide gel electrophoresis of Proteus flagellins at pH 2.7 in the presence of 8M urea revealed very little difference in mobility both within and between flagellins isolated from different Proteus species. This contrasted with results obtained by Barr (1973) using starch gel electrophoresis, although she concluded that the observed differences in mobility were not significant for purposes of phylogeny.

When Salmonella g,s,t flagellin was digested with trypsin, the number of tyrosine- and arginine-containing peptides equalled the number of residues obtained by amino acid analysis. This is to be expected if the similar residues are well distributed along the polypeptide chain. However, in the CNBr A and D fragments of flagellin, the number of tyrosine- and arginine-containing peptides exceeded that of the corresponding residues. This was probably due to degradation of the tryptic peptides resulting in weak-staining spots which were included in the peptide numbers. In contrast, the number of arginine- and tyrosine-containing peptides in 'C,D' complex were less than the corresponding number of residues. This was probably due to incomplete trypsin digestion; ninhydrin-positive material was invariably observed at the origin of the peptide map.

In the whole flagellin, the total number of tryptic peptides was slightly less than the summed numbers of lysine and arginine residues as determined by amino acid analysis, whereas in the CNBr fragments it was slightly more. Again, this is probably explained by incomplete tryptic digestion, and degradation of tryptic peptides, respectively.

Although it was not possible to determine the amino acid sequences of the g,s,t CNBr B and D tryptic peptides, the close similarity in amino acid composition of some of these peptides with those obtained by Joys and Rankis (1972) from S. typhimurium H1-i flagellin, justifies the assumption that the sequences are also similar. Seven peptides are homologous in the two proteins as judged by their amino acid compositions. A tryptic peptide from g,s,t, CNBr D fragment with the sequence "Ser - Arg" is also present in flagellins of S. typhimurium (TP26); S. adelaide, peptide Z1d of CNBr fragment D, (Davidson, 1971); and B. subtilis 168, residues 261 and 262 (DeLange et al., 1976). Other homologies between the sequences of these flagellins are shown in Figure 10. It is of note that these amino acid sequences are present at

FIGURE 10

Comparison of partial sequences of diverse flagellins. The sequences of the S. senftenberg g,s,t CNBr B and D tryptic peptides have not yet been determined. However, because their amino acid compositions are so very closely similar to those of certain S. typhimurium i peptides (Joys and Rankis, 1972), the assumption was made that their sequences are also very similar.

Residues identical in flagellins from 2 bacterial genera are underlined.
Double underlining indicates identities in the proteins from three genera.
Triple underlining indicates identities in the proteins from four genera.

* Glutamic acid and aspartic acid may be in the positions shown, or in the reverse positions.

	10
B. subtilis 168	Leu-Asn-Thre- Leu -Asn-Arg
S. typhimurium, TP18	Leu-Asn- Glu -Ileu-Asp-Arg
* g,s,t, CNBr B, T-1	Leu-Glu- Glu -Ileu-Asp-Arg
	30
B. subtilis 168	Leu-Ser-Ser-Gly-Leu-Arg
S. typhimurium, TP24	Leu-Ser-Ser-Gly-Leu-Arg
g,s,t, CNBr B, T-17	Leu-Ser-Ser-Gly-Leu-Arg
	90
B. subtilis 168	Val-Arg
S. typhimurium, TP28	Val-Arg
	233
B. subtilis 168	Ala-Lys-Leu-Gly-Ala- Val -Gln-Asn-Arg
S. typhimurium, TP10	Ser-Asp-Leu-Gly-Ala- Val -Gln-Asn-Arg
g,s,t, CNBr D, T-1	Ser-Asp-Leu-Gly-Ala-Ileu-Gln-Asn-Arg
	260
B. subtilis 168	Glu-Ser- Arg -Ileu-Arg-Asp-Val-Asp-
S. adelaide, Z1d	Ser- Arg -Ileu-Glu-Asp-Ala-Asp-
B. subtilis 168	Met-Ala- Lys -Glu-Met-Ser-Glu-Phe-Thre-Lys
S. adelaide, Z1d	Tyr-Ala-Thre-Glu-Val-Ser-Asn-Met- Ser -Lys
S. typhimurium, TP26	Ser-Arg
g,s,t, CNBr D, T-2	Ser-Arg

Figure 10

either the N- or C-terminal end of the flagellin molecule in support of the theory that the variable part of the molecule is the central region.

When the amino acid contents of the g,s,t CNBr B tryptic peptides (except peptides 10 and 19) are summed and compared to the amino acid analysis of the whole CNBr B fragment, one aspartic acid, one threonine, one alanine, two valines, three leucines, two phenylalanines and one lysine are missing. As only one lysine residue and no arginine residues are unaccounted for, it is likely that one large tryptic peptide was left at the origin of the peptide map. However more serine, glutamic acid and glycine residues are present in the summed amino acid contents of the peptides than in the amino acid analysis of the whole CNBr fragment. (Peptides 10 and 19 were not included as their amino acid composition results were felt to be unreliable.)

Although it was possible to obtain sufficient amounts of the peptides for amino acid analysis by eluting from peptide maps, the method was unsuitable when quantities sufficient for amino acid sequencing were needed. The use of DEAE-cellulose columns, for example, may prove suitable for the fractionation of tryptic peptides for sequencing purposes.

To investigate the serological differences between Salmonella g.... antigenic strains, antisera were prepared against g,s,t; g,m and g,p flagella. Single factor antisera were obtained by absorption of these antisera; these studies suggested the presence of a Z_8 antigenic factor in the g,m antigenic strain, which had not previously been reported. Although the single factor antisera had lower agglutination titres than the unabsorbed antisera from which they were prepared, as would be expected, the agglutination titres of certain single factor sera did not correspond very well with their immobilisation titres. This was also true in the case of anti-g,p serum. The reason for this is not

clear; the end-point of the immobilisation titre, however, was not so sharp as that of the agglutination titre which may have introduced some error into the titrations.

The ability of flagella and flagellin CNBr fragments to inhibit the activity of unabsorbed and single factor antisera were tested by two techniques; immobilisation- inhibition (II) and complement fixation-inhibition (CFI) assay. Using the former technique, anti-g,s,t was inhibited equally effectively by g,s,t CNBr A; g,m CNBr A and g,p CNBr A. However, g,m CNBr A was only a third as effective as g,s,t CNBr A as an inhibitor of anti-g,s,t, as measured by the latter technique. Assuming that the CNBr A fragment carries the antigenic determinants of the flagellin molecule (Parish, Wistar and Ada, 1969), and that the three g.... antigenic types have g subfactors in common, it would be expected that the unabsorbed sera would be inhibited by each of the three g.... antigenic CNBr A fragments; the homologous CNBr A fragment would be the most effective inhibitor. However, although this was true for anti-g,s,t, as shown most clearly by CFI, it was not found to be so in the case of anti-g,m. Although g,m CNBr A inhibited anti-g,m, neither g,s,t CNBr A or g,p CNBr A were able to block its immobilising activity. However, some inhibition of anti-g,m by g,s,t CNBr A was demonstrated using the CFI assay, although this was considerably less than the inhibition of anti-g,s,t by g,m CNBr A. Possibly the g subfactors of g,s,t flagellin common to g,m flagellin are not located on the CNBr A fragment, thus making g,s,t A an ineffective inhibitor of anti-g,m.

The CFI results suggested that g,s,t CNBr B was as efficient an inhibitor of anti-g,s,t as g,m CNBr A, which seems rather surprising. However, Parish, Wistar and Ada (1969) have reported antigenic activity in the CNBr B fragment of S. adelaide flagellin using the haemagglutination-inhibition test; this activity was not detected by

bacterial immobilisation, immunodiffusion or quantitative microprecipitation. The results obtained for anti-g,p serum using the II technique also indicated a lack of antigenic activity in g,p CNBr B fragment. No antigenic activity has been detected in either g,s,t CNBr D (CFI) or g,p CNBr D (II) fragments. These results suggest that CFI is the more sensitive technique for determining the antigenicity of protein fragments as it is able to detect minor antigenic activities which remain undetected by II.

Neither g,m CNBr A or g,p CNBr A efficiently inhibited the ~~immobilising~~ activity of anti-t as measured by both II and CFI. However, g,s,t CNBr A was an effective inhibitor of anti-t. These findings are as expected as g,m CNBr A and g,p CNBr A do not carry a t antigenic factor, whereas g,s,t CNBr A does. Similarly, anti-s was effectively inhibited by g,s,t CNBr A but not by g,m CNBr A (CFI), and anti-p was effectively inhibited by g,p CNBr A but not by g,s,t or g,m CNBr A fragments (II). These latter results were not confirmed by CFI, which showed no inhibition of anti-p by any of the CNBr A fragments. However it is likely that g,p CNBr A fragment would have inhibited activity if higher concentrations had been used.

The results obtained for anti-m serum were rather surprising. Not only g,m CNBr A, but g,p CNBr A, and to a lesser extent, g,s,t CNBr A were effective as inhibitors of immobilising activity. Although the two latter CNBr A fragments do not carry an m antigenic factor, their conformation must be such that they can interact with and inhibit anti-m activity when the antibodies to the g subfactors of g,m are absent. If the g subfactors of g,s,t CNBr A and of g,p CNBr A are different to those of g,m flagella then anti-g would presumably prevent their interaction with anti-m in the anti-g,m serum.

In each case, homologous flagella were slightly more effective inhibitors of immobilising activity than the homologous CNBr A fragment.

This could be due to a loss of antigenicity during isolation of the CNBr fragments or it might indicate that not all the antigenic determinants are located on CNBr A fragment.

If supplies of antisera had allowed, the range of inhibitors tested against each antiserum would have been extended to include flagella, flagellins and CNBr B and D fragments of both homologous and non-homologous g.... antigenic types.

In the determination of the sequence basis of serological differences between g.... antigenic flagellins, comparison of the amino acid compositions of CNBr A fragments has only a limited use. Unless antigenic differences are due to differences in amino acids which are present in small amounts, it would be difficult to distinguish them from the differences inherent in the amino acid analysis of a peptide of this size. Simon et al. (1977) divided B. subtilis flagellins into four groups on the basis of their amino acid compositions. These groupings correlated well with the serological classification of the flagellins. These workers noted that the difference in aromatic amino acid numbers were most pronounced and suggested that phenylalanine and tyrosine were important in determining the antigenicity of the molecule. When stains specific for tyrosine (and arginine) residues were applied to tryptic peptide maps of g,s,t; g,m and g,p flagellins, no difference in the distribution or number of tyrosine residues were observed.

Earlier, Ichiki and Parish (1972) had compared the amino acid compositions of CNBr fragment A of S. adelaide f,g flagellin and the antigenically active tryptic and peptic peptides isolated from it, and concluded that the methionine and arginine residues of flagellin did not play an important antigenic role. Interestingly, the enzyme-resistant peptides contained a comparatively high proportion of the NML, threonine and phenylalanine residues present in flagellin.

Work was started on isolating a trypsin-resistant core from g,s,t flagellin in an attempt to locate the antigenic site more precisely. When dialysed tryptic digests of g,s,t flagellin or g,s,t CNBr A fragment were run on pH 2.7 polyacrylamide gels, two bands with similar electrophoretic mobility, running faster than CNBr A, were observed. Thus the undigested core must have been derived from the antigenic CNBr A region of the flagellin molecule. Similar results were obtained with g,m CNBr A and g,p CNBr A. An attempt to separate the two peptides on Sephadex columns was unsuccessful; ion-exchange chromatography will probably prove useful for this purpose.

Peptide differences between the g.... antigenic flagellins were revealed by enzymic digestion followed by a combination of chromatography and electrophoresis. Thermolysin and chymotrypsin proved useful in this respect. In each case, the peptide differences were located on the CNBr A fragments of the flagellins, as would be expected, but the amino acid substitutions responsible for them have not yet been identified.

Genetic analyses by P22 phage-mediated transduction in Salmonella indicated that all mutant sites responsible for flagellar shape are inseparable from a structural gene of flagellin, H1 or H2; depending on which gene mutated, the mutant shape appeared in either phase 1 or phase 2 (Iino, 1962; Iino and Mitani, 1966). A difference in 1 peptide among 35 between normal flagellin and flagellin from a curly mutant of S. abortusequi were observed on enzymic peptide maps (Enomoto and Iino, 1966). A more detailed comparison of the chemical compositions of the flagellin of normal and straight flagella of B. subtilis (Martinez et al., 1968) revealed that, in flagellin of straight mutants, an alanine molecule in normal flagellin is replaced by valine in the altered peptide.

Curly mutant sites in Salmonella were genetically mapped in a region proximal to the N-terminus (Iino, 1977); the straight mutant site of B. subtilis was chemically identified to be close to the C-terminus. Thus the genetic change of flagellar shape is attributed to a mutation at a site in a structural gene of flagellin which results in the replacement of a particular amino acid in the flagellin polypeptide by another specific one. The flagellin polypeptide with the altered amino acid sequence may then undergo folding to form an altered conformation of monomer, and consequently the mode of polymerisation of the flagellin may be changed. The primacy of the type of monomer for flagellar shape was also shown by reconstitution of flagellar fibres from flagellin: the overall shape of the reconstituted fibres was found to be predominantly determined by the nature of the monomer (Asakura, Eguchi and Iino, 1966).

The amino acid substitutions involved in the morphological mutants of Salmonella g.... antigenic strains proved to be difficult to identify, despite the use of a range of enzymes.

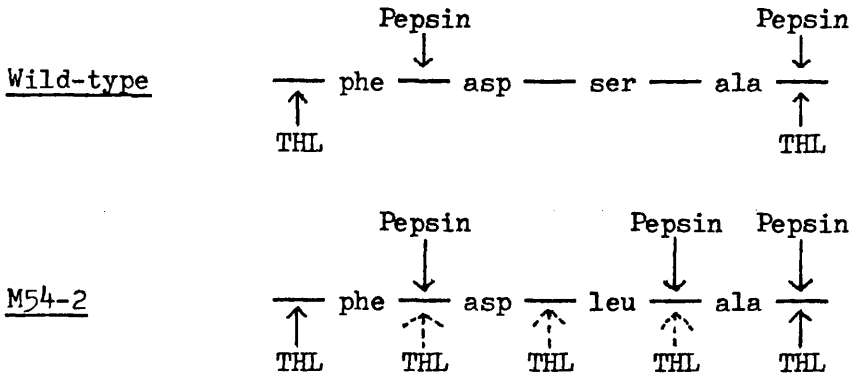
A tyrosine-containing tryptic peptide in f,g D24-2 (curly II) flagellin was observed to move much more slowly chromatographically than the corresponding peptide in the wild-type f,g flagellin. The same peptide in wild-type g,s,t flagellin had been located in CNBr fragment D. Although the actual amino acid substitution has not yet been determined, it can be surmised from the change in position of the peptide that a hydrophobic amino acid in the wild-type flagellin has been substituted for in the mutant flagellin.

Peptide differences between g,s,t M54-2 (curly II) and wild-type g,s,t flagellins have been observed on both thermolytic and peptic peptide maps:

An acidic thermolytic peptide present in wild-type g,s,t flagellin was shown to be absent from g,s,t M54-2 flagellin; no replacement peptide

was detected in the mutant flagellin. Thermolytic peptide maps of CNBr fragments of g,s,t mutant flagellins suggest that the peptide is located in CNBr B or D. However, the peptide was also absent from flagellin thermolytic peptide maps of "revertants" of the g,s,t M54-2 mutant strain, suggesting that either these were not true revertants or that the peptide difference is not involved in the change of waveform of the flagella. The wild-type peptide was composed of a residue each of aspartic acid, serine, alanine and phenylalanine. As thermolysin preferentially hydrolyses the bond in which the NH_2 group is donated by a hydrophobic amino acid phenylalanine is probably the N-terminal amino acid of the peptide.

On the peptic peptide maps, a peptide present in the wild-type g,s,t flagellin moved much faster chomatographically in the g,s,t M54-2 flagellin. The wild-type peptide was composed of a residue each of aspartic acid, serine and alanine. Serine was replaced by the more hydrophobic leucine in the mutant peptide, thus causing the peptide to move faster chromatographically. Sometimes two spots were observed instead of one on the mutant peptide map. The slightly slower running spot, chromatographically, consisted of aspartic acid, leucine and alanine; the other spot consisted of aspartic acid and leucine only. It seems that the substitution of leucine for serine in the mutant flagellin created a further cleavage site for pepsin. If a single base change is assumed, the substitution of leucine for serine is the result of a transition from C to U in the second base of the triplet codon UCA/G of serine. Knowing the specificities of the enzymes, thermolysin (THL) and pepsin, an amino acid sequence for the wild-type g,s,t and g,s,t M54-2 peptides was proposed:



Thus it is now possible to suggest why no replacement peptide for the wild-type peptide was observed on thermolytic peptide maps of g,s,t M54-2 flagellin. The dotted arrows indicate the possible sites of thermolytic cleavage; the result of such cleavage would have been the release of free amino acids.

The presence of an amino acid, histidine, which is missing from wild-type g,s,t flagellin, was detected in the mutant g,s,t flagellins M32-2 and D10-2. Attempts to identify the histidine-containing peptides in the mutant flagellins have not yet been successful, but examination of their CNBr fragments will probably prove useful in this respect.

The C-terminal sequence of g,s,t M5-2 (curly IV), g,m D8-1 (straight) and g,s,t M55-3 (curly II) flagellins was determined as:

— ser — leu — leu — argCOOH

that is, identical to that of the wild-type g,s,t and g,m flagellins.

Although the results are limited, they do suggest that, in agreement with other workers' results, the sites of amino acid substitution in morphological mutant flagellins are located at the N- or C-terminal ends of the molecule. This corresponds to CNBr fragments B, and C and D, respectively. The C-terminus of the flagellin molecule appears to be conserved.

It is hoped that further work involving comparison of 'C,D' complexes of mutant flagellins with that of the wild-type will reveal

the amino acid differences which have, up to now, proved to be difficult to demonstrate. The reasons for this are not clear; it seems unlikely that conservative amino acid changes, which would be undetectable on peptide maps, would be enough to alter the conformation of the molecule. On the other hand, the substitution of a hydrophobic residue for a polar one, for example, should be easily detected on a peptide map. The fact that so few changes have been observed might suggest that they were so slight as to be attributed to the inherent variability of the method.

One of the many uses of chemical modification of specific amino acids in proteins is to distinguish the surface residues from those in the interior of the molecule. When the monomeric forms of various bacterial flagellins were modified with cyclohexanedione, more arginine residues were modified than when the polymeric forms were modified. This was shown both by the greater number of modified arginines and the smaller number of unmodified arginines present. However, the recovery yield of arginine residues in the modified monomer form of the flagellins was poorer than for the modified polymer form of most of the flagellins analysed. It was therefore assumed that the arginine residues not detected by amino acid analysis were probably modified, but were susceptible to degradation under the conditions of hydrolysis. Attempts to prevent this degradation were not successful.

In the monomer form of the flagellins, most of the arginine residues were accessible to the modifying reagent. In the polymer form, a higher proportion of arginine residues were unmodified in those flagellins with a higher total number of arginine residues. This implies that the additional residues in these flagellins are buried in the protein matrix. In each polymer, the number of modified arginine residues was at least one residue less than the number in the

corresponding monomer; in half of the flagellins examined the disparity was much greater. Thus the results suggest that at least one arginine residue becomes buried upon the aggregation of flagellin subunits into helical filaments.

The arginine residues in the flagellins analysed can, therefore, be divided into 3 subgroups on the basis of these chemical modification experiments: a very small number of residues inaccessible to the reagent; at least one, and probably more, residues which were buried in the polymer but not in the monomer; several residues which were modified in either case.

Comparison of the results obtained when monomeric, native (obtained by acid dissociation of a flagella pellet) and purified S. senftenberg flagellin (obtained by acid dissociation of a flagella pellet which had been precipitated with ammonium sulphate subsequent to acid dissociation) revealed very little difference in reactivity, as would be anticipated. However, more arginine residues were modified in the purified than in the native polymer, suggesting that the purification process had caused the exposure of certain arginine residues which, in the native polymer, would have been inaccessible to cyclohexanedione. As the other flagellins modified were purified preparations, it therefore seems likely that the number of arginine residues modified in the polymeric forms is higher than would have resulted from modification of native flagella. In turn, this means that the difference between the number of modified arginine residues present in the monomeric and polymeric forms would be greater. The minimum estimate of one arginine residue becoming buried on polymerisation is probably, therefore, a substantial underestimation.

The distribution of arginine residues within the flagellin molecule is of particular interest. One of the flagellins modified, that of B. subtilis 168, has had its complete amino acid sequence

determined (DeLange et al., 1976). Although hydrophobic residues are distributed randomly, the distribution of charged residues is strikingly asymmetric. The NH_2-terminal region possesses a net charge of 6 plus; the middle of the molecule, a net charge of 9 minus and the COOH-terminal region, a net charge of 4 minus. There is only one arginine residue present in the middle of the molecule. Similarly, only one arginine residue was detected in the CNBr A fragment of S. senftenberg g,s,t flagellin. (Parish and Ada (1969) and Davidson (1971) established the sequence of CNBr fragments in S. adelaide flagellin, placing the CNBr A fragment in the middle of the flagellin molecule.) In contrast, 6 arginine residues were detected in the P2 peptide of P. mirabilis flagellin (Glossman and Bode, 1972) which corresponds to the A plus D CNBr fragment of S. adelaide flagellin. However, the distribution of these residues is not yet known; it would be expected that they are located at the extremities of the P2 peptide.

The other 14 arginine residues of B. subtilis flagellin are situated at the NH_2- and COOH- terminal regions of the molecule. Similarly in S. senftenberg g,s,t flagellin, 10 arginine residues are located in CNBr B, and CNBr C and D fragments which were identified as the NH_2- and COOH- terminal regions respectively in S. adelaide flagellin (Parish and Ada, 1969).

It is of note that 5 of the arginine residues in B. subtilis flagellin have been identified in homologous amino acid sequences in S. typhimurium and S. senftenberg flagellins. (Although the tryptic peptides of S. senftenberg were not actually sequenced, their amino acid compositions were so similar to corresponding tryptic peptides of S. typhimurium that the assumption was made that the sequences were also homologous). In addition, arginine residue 262 of B. subtilis flagellin has also been identified in a homologous amino acid sequence in S. adelaide flagellin. All four flagellins have

arginine as their COOH-terminal residue.

Thus a high proportion of the arginine residues are located in amino acid sequences which are probably conserved, situated at the N- and C-terminal ends of the flagellin molecule. It seems likely, therefore, that they are necessary for the proper folding and aggregation of the flagellin subunits to form the stable helical flagella.

When S. senftenberg g,s,t monomeric flagellin was nitrated with tetranitromethane, 6 of the 7 tyrosine residues did not react with the reagent. One explanation for these results is that the flagellin polypeptide chain is tightly folded causing the tyrosine residues to be buried in the protein structure. If so, these tyrosine residues should become accessible to the modifying reagent by treating the protein with urea or guanidine hydrochloride, which would cause the polypeptide chain to unfold.

In the polymer, all 7 tyrosine residues remained unmodified; that is, the tyrosine residue modified in the monomer was no longer accessible to the reagent in the polymer. This suggests that a change in conformation has taken place during polymerisation. This is consistent with Asakura's (1968) two step concept of polymerisation: the first step is the reversible binding of a monomer to an end of an existing filament; the second is the incorporation of the bound monomer into the filament, accompanied by its conformational change.

When S. paratyphi B b flagellin was nitrated, one tyrosine residue in the monomer became masked in the polymer, but, in addition, two residues were accessible to the reagent in either case. Similarly, the 6 tyrosine residues of B. stearothermophilus flagellin was divided by Smith and Koffler (1971) into 3 subgroups according to nitration experiments only: three phenolic groups were not accessible to modification; two were buried upon aggregation, whereas the sixth

tyrosine was nitratable in either case. Spectrophotometric experiments indicated that in the flagellin of S. typhimurium only 2 of the 9 phenolic groups can be normally ionised, but none in the polymeric flagellum (Taniguchi, 1970). Schalch and Bode (1975) presented evidence from both chemical modification and spectral studies that 2 tyrosine residues were unmasked in the isolated P. mirabilis flagellin. Aggregation experiments indicated that both of these residues may be involved in the activation step of the aggregation and/or even in the stabilising interactions in the polymer structure. In the polymer, both the tyrosine residues became inaccessible to solvent reagents after specific aggregation. Four of the five tyrosine residues of P. mirabilis flagellin are located in the P2 fragment in the centre of the molecule, the fifth tyrosine being present in the N-terminal P3 fragment. Similarly, four tyrosine residues of S. senftenberg g,s,t flagellin are present in the CNBr A fragment in the centre of the molecule, and two in the 'C,D' complex at the C-terminal end of the molecule.

Tyrosine residues in E. coli flagellin were not modified in either the monomer or polymer form of the flagellin. It is also of interest that several bacterial flagellins possess only one or two tyrosine residues, for example, B. subtilis, B. sphaericus, B. pumilis, Erwinia carotovora, Ps. aeruginosa and R. rubrum flagellins. Thus the suggestion made by Schalch and Bode (1975) that at least two tyrosines, which become buried during the flagella formation, play an essential rôle for the structural and functional requirements of the bacterial flagella may only apply to certain flagellins.

The exact location of amino acids essential for the structural and functional requirements of bacterial flagella have still to be determined. Their identification will require a prior knowledge of the amino acid sequence of the flagellin molecule.

Comparison of the amino acid compositions of various bacterial flagellins shows that they all have high proportions of aspartic and glutamic acids (or amides) and alanine, but small amounts of proline, methionine, tyrosine, phenylalanine and histidine. Methionine was present in all the flagellins analysed except that of Alcaligenes faecalis. This flagellin also lacked histidine, as did that of Erwinia carotovora, all the Salmonella g...antigenic strains except possibly g,q, and some Proteus strains. It was noted that in the Bacillus flagellins there was only one tyrosine residue, but approximately 10 methionine residues. This was also true for E. carotovora, Ps. aeruginosa and R. rubrum flagellins. In contrast other flagellins such as those of E. coli K12, S. marcescens and Salmonella g...antigenic strains contained twice as many tyrosine as methionine residues, the total number of methionine plus tyrosine residues being approximately the same. In P. mirabilis, P. vulgaris, P. rettgerri, P. morganii and Ps. fluorescens flagellins, the number of tyrosine and methionine residues was about equal. If hydrophobic bonding plays an important part in the stabilisation of flagellin-flagellin interactions, then the proportions of tyrosine and methionine residues may be of significance.

Various approaches have been used to study the phylogenetic relationships between bacteria, including estimations of their mole % guanine plus cytosine (G. C.) content (Marmur, Falkow and Mandel, 1963), 16 S ribosomal RNA sequence characterisation (Fox et al., 1980), and now, comparison of bacterial flagellin amino acid sequences. It was noted that the flagellin amino acid compositions of bacteria with markedly different mole % G.C. contents varied significantly. However, the flagellin amino acid compositions of bacteria with G.C. contents in the middle of the range did not differ to any great extent. This is not altogether surprising as these were comprised mainly of bacteria

of the Enterobacteriaceae family such as E. coli K12, Salmonella and S. marcescens, which would be expected to have similar amino acid compositions. Strains of Bacillus, the only Gram positive organisms studied, had very similar GC contents to those of Proteus; their corresponding flagellins had very different aromatic amino acid compositions.

The information obtained from amino acid analysis, chemical modification, morphological mutants and serological studies of the Salmonella g.... antigenic flagellins, is consistent with the theory that the N- and C-terminal sequences of the flagellin molecule are conserved and, therefore, contain the residues important for the proper folding and aggregation of the flagellin subunits to form functional, helical flagella. Peptide maps of flagellins isolated from E. coli, and from antigenic types of Salmonella other than the g.... antigenic strains have indicated the presence of tryptic peptides which are known to be located in the CNBr B fragment of S. senftenberg g,s,t flagellin. The CNBr B fragment is positioned at the N-terminus of the molecule. On the other hand, the central part of the molecule, represented by the CNBr A fragment in S. senftenberg g,s,t flagellin, appears to be the variable region in which amino acids are substituted, deleted or inserted. The remarkable variation in the antigenic types of Salmonella, E. coli and B. subtilis flagellins is thus manifested by amino acid changes in this region of the molecule. This corresponds with Shirakihara and Wakabayashi's model in which the antigenic region is represented by a surface bead (region S) which does not contribute greatly to the intersubunit bonds, so that any change that occurs in that outer bead would not affect the packing pattern of the flagellin molecule very much, but could affect the antigenicity of the flagella.

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