Flagellar Synthesis in *Salmonella typhimurium*: the Incorporation of Isotopically-Labelled Amino Acids into Flagellin

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SUMMARY

A method is described for assaying flagella of *Salmonella typhimurium* based on the incorporation of a ^{14}C -labelled amino acid, mechanical separation of the flagella from the bacteria, and measurement of the radioactivity of the isolated flagella. There is no detectable lag before the appearance of L-[$G\text{-}^3\text{H}$]leucine, or L-[$Me\text{-}^{14}\text{C}$]methionine in the flagella, and the rates of incorporation of these amino acids into flagella paralleled the rates of incorporation into the general cell protein. Mechanical removal of the flagella did not affect their subsequent rate of formation. The amino acid, $\epsilon\text{-N}$ -methyllysine, present in flagellin, can be labelled with either L-[$U\text{-}^{14}\text{C}$]lysine or L-[$Me\text{-}^{14}\text{C}$]methionine. Using $\epsilon\text{-N}$ -methyllysine as a marker, it has not been possible to detect soluble protein precursors in the formation of flagella by *S. typhimurium*.

INTRODUCTION

Although the flagella of *Salmonella typhimurium* are well defined serologically and chemically (Kauffmann, 1954; Ambler & Rees, 1959) and are probably a pure protein named flagellin, studies on their formation have been hampered by the absence of a specific quantitative assay for flagellin. Stocker & Campbell (1959) followed the synthesis of flagella by measuring the total length of flagella in cultures of *S. typhimurium* and found that flagellin behaved as a normal constitutive protein. Mechanical removal of flagella by treating bacteria in a Waring blender had no effect on the subsequent rate of formation of flagella. Weinstein, Koffler & Moskowitz (1960) reported the presence of flagellin in the soluble protein fraction obtained from lysed spheroplasts of *Proteus vulgaris*. This intracellular flagellin was detected and assayed serologically and amounted to about 1 % of the total flagellin of the culture. The origin of the intracellular material was not determined so it may have arisen during breakage and fractionation of the cells. However, the report of flagellin in the soluble protein fraction of *P. vulgaris* raises the possibility that soluble protein precursors are synthesized during the formation of functional flagella.

The present paper describes possible specific assays of flagella and attempts to demonstrate intermediates in the formation of flagella by *Salmonella typhimurium*. A preliminary report of this work has been given (Kerridge, 1961*a*).

METHODS

Organism and growth media. The organism used throughout this work was *Salmonella typhimurium*, strain sw1061, obtained from Dr T. Iino (National Institute of Genetics, Misimo, Shizuoko-ken, Japan). The culture was maintained on Dorset egg slopes at room temperature. For the preparation of washed suspensions the bacteria were grown at 37° in Roux bottles containing 150 ml. Oxoid nutrient broth, or in 250 ml. conical flasks containing 100 ml. buffered salts solution (DO)+0.2 % (w/v) glucose (Kerridge, 1959). The bacteria were harvested while still in the exponential phase of growth, washed once with saline (0.85 %, w/v, NaCl) or with distilled water and resuspended at a concentration equivalent to 5 mg. dry wt. organism/ml. Non-flagellated cultures of *S. typhimurium* were obtained by mechanical removal of the flagella (Kerridge, 1959) or by growth of the bacteria at 44° (Kerridge, 1961*b*).

The dry weights of bacteria in suspension were determined from optical density measurements made with a Hilger 'Spekker' absorptiometer and the use of a calibration curve prepared by drying bacterial suspensions of known optical density to constant weight at 105°.

Incorporation of ¹⁴C-labelled amino acids into Salmonella typhimurium. The incorporation of ¹⁴C-labelled amino acids into suspensions of *S. typhimurium* was carried out in DO medium containing 0.2 % (w/v) glucose; the ¹⁴C-labelled amino acid was added to give a final concentration of 0.1 µC./ml., although the specific activity varied from experiment to experiment. In certain experiments this medium was supplemented with a mixture of 18 amino acids each at a final concentration of 0.05 mg. of L-isomer/ml. The bacteria were added to a final concentration equiv. 0.5 mg. dry wt./ml. and the suspensions incubated statically at 37°. Occasionally the labelled amino acid was added to cultures of *S. typhimurium* growing exponentially in defined media.

At intervals during incubation, samples containing equiv. 2-3 mg. dry wt. bacteria were pipetted into centrifuge tubes containing sufficient chloramphenicol to give a final concentration of 50 µg./ml. and the tubes cooled to 0°. The bacteria were harvested by centrifugation and, after washing once with cold distilled water, were resuspended in 2.5 ml. distilled water. The flagella were detached mechanically and separated from the bacteria by centrifugation at 5000*g* for 15 min.

Isolation and estimation of ¹⁴C-labelled flagella. A preparation of unlabelled flagella was added to the supernatant fluid containing the ¹⁴C-labelled flagella to a final concentration of 0.1 mg. protein/ml. and the flagella precipitated from this suspension by using the techniques described below:

(a) *Precipitation with acriflavin.* This method was derived from the procedure of Bernstein & Lederberg (1955). An aqueous solution of acriflavin was added to the suspension of flagella to a final concentration of 0.05 or 0.1 % (w/v). The tubes were incubated at 37° for 2 hr. and then at 0° for 16 hr. The agglutinated flagella were harvested by centrifugation and the radioactivity determined.

(b) *Precipitation with anti-flagellar antiserum.* A volume of 0.04 ml. anti-1.2.3. antiserum (titre 1/10,000; obtained from Dr P. Bradstreet, Serological Standards Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9), was added to 2.0 ml. of the flagellar suspension. The tubes were

incubated at 37° for 2 hr. and then at 0° for 16 hr. The antigen + antibody complex was recovered by centrifugation, washed once with saline, and resuspended in 1 ml. 5 % (w/v) trichloroacetic acid (TCA). The precipitate was washed once with 5 % (w/v) TCA and then taken up in $N-NH_4OH$ for the assay of radioactivity.

(c) *Precipitation with TCA.* TCA was added to the flagellar suspension to a final concentration of 5 % (w/v) and the suspension stored at 4° for 30 min. After centrifugation and washing with 5 % (w/v) TCA, the precipitate was resuspended in 2 ml. 6 $N-HCl$ and hydrolysed at 105° for 16 hr. The HCl was removed by distillation and samples of the hydrolysate equivalent to 0.1 mg. protein were subjected to electrophoresis on Whatman no. 3MM paper in 0.05M sodium carbonate + bicarbonate buffer (pH 10.6) for 2 hr. at 20 V./cm. The amino acids were located by dipping the electrophoresis paper in an acidic solution of ninhydrin (ninhydrin, 0.5 g.; acetone, 100 ml.; glacial acetic acid, 30 ml.). The areas corresponding to ϵ -*N*-methyllysine (NML) and lysine were cut out and the amino acid-ninhydrin complex eluted with water. The eluates were transferred quantitatively to aluminium planchets (8 cm.²) and the radioactivity determined with a thin window gas flow counter (Nuclear Chicago Corp., 333 East Howard Avenue, Des Plaines, Illinois, U.S.A.).

Cell fractionation. The procedure was based on that described by Roberts *et al.* (1955). The deflagellated bacteria were washed once on the centrifuge with distilled water and the pellet resuspended in 2 ml. 5% (w/v) TCA. After storage at 4° for 2 hr., the precipitated bacteria were harvested by centrifugation and washed once with 5 % (w/v) TCA. The precipitate was extracted for 30 min. at 45° with 2 ml. 75 % (v/v) ethanol in water, the suspension centrifuged, and the pellet extracted for 30 min. at 45° with a mixture of 1 ml. 75 % (v/v) ethanol in water + 1 ml. ether. The ethanol and ethanol + ether extracts were combined and, after the addition of 3 ml. water, were extracted three times with an equal volume of ether to remove lipids. Nucleic acids were extracted from the residue after ethanol + ether extraction with 5 % (w/v) TCA, one extraction at 90° for 30 min. being sufficient to remove the nucleic acid. The TCA-insoluble residue contained the residual protein and cell-wall fractions.

The protein-containing fractions were hydrolysed with 6 $N-HCl$ at 105° for 16 hr. After removal of HCl , the hydrolysate was dissolved in distilled water and samples taken for electrophoresis and for radioactivity assay. Samples were transferred to aluminium planchets (2 cm.²) and the radioactivity determined using a Panax type D657 Scaler (Panax Equipment Ltd., Mitcham, Surrey) in conjunction with a mica end-window Geiger-Muller tube.

Incorporation of L-[G-³H]leucine into Salmonella typhimurium. L-[G-³H]leucine was used in one experiment to follow protein synthesis in *Salmonella typhimurium*. The incubation conditions and fractionation procedure were identical to those used to study the incorporation of ¹⁴C-labelled amino acids by *S. typhimurium*. After fractionation the TCA insoluble residues were dissolved in 0.3 ml. of a M solution of Hydroxide of Hyamine 10- $\times$ (Reg. trademark of Rohn and Haas Inc.) in methanol. An aliquot of this solution was added to 1.5 ml. of a liquid scintillator, consisting of 3.5 g. 2,5-diphenyloxazole (PPO) and 50 mg. 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP) in 1 l. redistilled toluene, contained in a small glass vial. The radioactivity was measured using a Packard Tricarb Liquid Scintillation Spectrometer (Packard Instrument Company, Inc., La Grange, Illinois, U.S.A.).

Disrupted cell preparations. Suspensions of *Salmonella typhimurium* were disrupted by using: (a) the Mullard type E 7590B ultrasonic disintegrator and the transducer assembly described by Davies (1959); (b) a bacterial press (Hughes, 1951); (c) a French pressure cell based on a design supplied by the Department of Terrestrial Magnetism, Carnegie Institute, Washington, D.C., U.S.A. The disrupted bacteria were fractionated by centrifugation at 5000g for 15 min., followed by 105,000g for 120 min., into a fraction containing unbroken bacteria and large particles, a fraction containing mainly ribosomes, and a soluble fraction.

RESULTS

Assay of bacterial flagella. A major difficulty in studying flagellar synthesis is that, up to now, there have been no sensitive methods for quantitative assay of bacterial flagella. Qualitative assays are relatively simple using microscopic examination of living cultures or stained bacterial preparations. In the absence of any specific enzymic activity in the flagellum, a study has been made of the possibility of isolating small quantities of radioactive flagella after their separation from the bacteria. Bernstein & Lederberg (1955) in confirming earlier studies of Sertic & Boulgakov (1936) on the agglutination of salmonella by acridines, reported that it resembled the agglutination of motile bacteria by specific anti-flagellar antiserum and that acridines agglutinated suspensions of isolated flagella. Flagella in phase 2 were agglutinated at a smaller concentration of the acridines than were flagella in phase 1. Addition of acriflavin (final concentration 0.05 % or 0.1 %, w/v) to a suspension of ³⁵S-labelled flagella (flagellar antigen 1.2.3.), kindly provided by Dr R. P. Ambler (Department of Biochemistry, Cambridge) precipitated 80 % of the added flagella (Table 1). Although acriflavin at a fairly high concentration gave good and reproducible precipitation of a purified preparation of flagella, this method was abandoned in favour of precipitation by specific anti-flagellar antiserum.

Table 1. *Precipitation of ³⁵S-labelled flagella of Salmonella typhimurium with acriflavin*

Suspensions of flagella were incubated with acriflavin for 2 hr. at 37° and then stored for 16 hr. at 4°. The precipitates were harvested by centrifugation, resuspended in N-NH₄OH and the radioactivity determined.

Acriflavin (%, w/v)	Flagella (mg./ml.)		
	0.2	0.1	0.05
	Radioactivity in the precipitate % of the total		
0.1	67	81	82
0.05	77	61	74

Under the experimental conditions used, addition of the specific anti-flagellar antiserum did not completely precipitate the ³⁵S-labelled flagella (Table 2a) for not more than 75 % of the added radioactivity was recovered. The results however, were very reproducible (Table 2b). Addition of anti-*i* antiserum to the suspension of ³⁵S-labelled flagella lacking this antigen did not cause the formation of any specific complex. In all subsequent serological isolations of isotopically-labelled

flagella, carrier flagella were added to give a final concentration of 0.1 mg. protein/ml. and specific anti-flagellar antiserum (titre 1/10,000) added at a dilution of 1 part in 50.

The incorporation of amino acids into flagella by Salmonella typhimurium. Stocker & Campbell (1959), in a study of the regeneration of flagella by mechanically deflagellated bacteria, found that the increase in total flagellar length, measured in stained preparations, agreed very well with theoretical values calculated on the assumption that flagellin behaved as a constitutive protein. These findings have been confirmed by studying the incorporation of L-[U-¹⁴C]leucine and L-[U-¹⁴C]-glutamic acid by cultures of *S. typhimurium* growing exponentially in defined media

Table 2. *Precipitation of ³⁵S-labelled flagella of Salmonella typhimurium with specific anti-flagellar antiserum*

Specific anti-flagellar antiserum (titre 1/10,000) was added to the flagellar suspensions, the preparations incubated at 37° for 2 hr. and then stored at 4° for 16 hr. The precipitate was removed by centrifugation, washed with saline and TCA (5%, w/v), finally suspended in N-NH₄OH and the radioactivity determined. 92% of the radioactivity in the flagellar preparation was precipitated with 5% (w/v) TCA.

	(a) Flagella (mg./ml.)			
	0.167	0.125	0.083	0.042
Serum dilution	Radioactivity in the precipitate, % of the total			
1/60	64	67	72	67
1/120	38	43	52	62
1/180	32	36	42	60
1/240	24	28	33	48

	(b) Flagella (mg./ml.)			
	0.2	0.1	0.05	0.025
Serum dilution	Radioactivity in the precipitate, % of the total			
1/50	57	72	74	.
	48	73	75	.
	57	75	74	.
	.	73	73	73

at 37°. Samples were taken at intervals after adding the amino acid, the bacteria washed once with saline and resuspended in 2.5 ml. saline. The flagella were then removed mechanically and separated from the bacteria by centrifugation. The detached flagella were precipitated with specific antiserum and the radioactivity in the precipitated flagella and the residual cell protein determined. The results are shown in Figs. 1*a* and *b*. There was no difference in the initial rates of incorporation of ¹⁴C-labelled amino acid into flagella and into the residual cell protein. Mechanical deflagellation immediately before the addition of the ¹⁴C-labelled amino acid had no effect on the relative rates of subsequent incorporation into flagella and residual cell protein.

Growth of *Salmonella typhimurium* at 44° in a complex medium leads to production of non-flagellated bacteria (Quadling & Stocker, 1956, 1962; Kerridge, 1961a). If the incubation temperature is lowered from 44° to 37° there is no change in the growth rate of the bacteria and the synthesis of flagella begins after a lag of about one generation time (Kerridge, 1961a). Addition of L-[U-¹⁴C]leucine to the culture immediately after transfer from 44° to 37° led to immediate appearance of ¹⁴C in the protein fraction of the bacteria and, after a lag, in the flagella. The rate of incorporation of the leucine in flagella was similar to the rate of incorporation in cell protein (Fig. 2).

Fig. 1a

Fig. 1b

Fig. 2

Figs. 1a, b. Incorporation of L-[U-¹⁴C]leucine and L-[U-¹⁴C]glutamic acid into *Salmonella typhimurium*. L-[U-¹⁴C]leucine or L-[U-¹⁴C]glutamic acid (final conc. 0.1 μ C./ml.) was added to a culture of *S. typhimurium* growing exponentially in the minimal medium supplemented with a mixture of 18 amino acids (50 μ g. of each/ml.). Samples were taken at intervals. The flagella were detached mechanically from the bacteria and precipitated serologically. The incorporation of the amino acid into flagella (-●-●-) and the acid-insoluble residue (-○-○-) was measured.

Fig. 2. Incorporation of L-[U-¹⁴C]leucine into *Salmonella typhimurium* during recovery of motility at 37° after growth at 44°. *S. typhimurium* growing exponentially at 44° in the minimal medium supplemented with 0.2% (w/v) Difco vitamin-free casein hydrolysate was harvested by centrifugation and resuspended in the minimal medium supplemented with a mixture of 18 amino acids (50 μ g. of each/ml.) and L-[U-¹⁴C]leucine (0.1 μ C./ml.) and incubated aerobically at 37°. After sampling, the flagella were detached mechanically from the bacteria and precipitated serologically. The incorporation of L-[U-¹⁴C]leucine into flagella (-●-●-) and the acid-insoluble residue (-○-○-) was measured. The figures in parentheses show % flagellate bacteria determined by microscopic examination of stained bacterial preparations.

Although in the early studies, the formation of flagella by *Salmonella typhimurium* has been considered as an example of the synthesis of a single protein, it is important to remember that the flagella of this organism are organized structures (Kerridge, Horne & Glauert, 1962). The relative rates of incorporation of isotopically-labelled amino acids into flagella and the general cell protein show that flagellin is a constitutive protein, but give no indication of possible protein intermediates between free amino acids and the functional flagellum. If such intermediates exist, they should be detectable by suitable isotopic techniques.

No evidence was obtained for the existence of a precursor pool from experiments

involving the addition of 200 times excess of unlabelled leucine to a culture of *Salmonella typhimurium* growing exponentially in a defined medium containing L-[U-¹⁴C]leucine. The incorporation of L-[U-¹⁴C]leucine into the residual protein fraction and the flagella ceased after adding the unlabelled amino acid (Fig. 3). However, this technique would only reveal a large, not a small pool of protein intermediates. The possibility that a small pool exists was next studied using high specific activity L-[G-³H]leucine. Samples were taken within 2 min. of adding leucine, but extrapolating to zero time gave no indication of a lag in the incorporation of tritium into flagella (Fig. 4) and therefore it is unlikely that a precursor pool capable of supporting more than a few sec. synthesis of flagella is present in *S. typhimurium*.

Table 3. *Distribution of flagellin in the soluble protein fraction of Salmonella typhimurium*

Bacterial suspensions were disrupted mechanically and the presence of flagellin in the 100,000g supernatant fraction detected serologically.			
Growth temp. (°)	Motility	Method of cell breakage	Flagellin in the 100,000g supernatant fluid
37	+	French pressure cell	+
37	—	French pressure cell	+
	(Flagella detached mechanically)		
44	—	French pressure cell	—
37	+	Hughes press	+
37	+	Lysis of penicillin-induced spheroplasts	+
Control flagellar suspension		French pressure cell	+

Serological examination of the soluble protein fraction of Salmonella typhimurium for the presence of flagellin. Weinstein *et al.* (1960) reported that flagellin could be detected in the soluble protein fraction of *Proteus vulgaris*. Attempts were made to repeat this finding with *Salmonella typhimurium* and to extend it by including methods of cell breakage other than lysis of penicillin-induced spheroplasts. Suspensions of *S. typhimurium* were disrupted mechanically by treatment in a bacterial press (Hughes, 1951), in a French pressure cell, and by the lysis of penicillin-induced spheroplasts. The broken cell preparations were centrifuged at 105,000g for 120 min. and the supernatant fraction freeze-dried. The soluble proteins were resuspended in saline (0.85 %, w/v) and examined serologically by the agar diffusion technique (Ouchterlony, 1953); the results are shown in Table 3. In all cases where flagellated bacteria were disrupted mechanically, flagellin was detected in the soluble protein fraction. A control experiment in which a suspension of isolated flagella, at a concentration equivalent to that present in the bacterial suspension, was 'disrupted' in a French pressure cell and examined serologically after centrifugation at 105,000g for 120 min. cast some doubt on the validity of the results, since 'flagellin' could obviously have arisen during mechanical disintegration of the bacteria. Mechanical removal of flagella before breaking the bacteria did not eliminate 'flagellin' from the soluble protein fraction, but, as it is difficult to remove all the flagella by this procedure it is impossible to decide conclusively if the flagellin present in the soluble protein fraction is an artifact. If the flagellin

detected in the soluble fraction is assumed to be a precursor for flagella, then the amount present (about 1 % of the total flagellin) is sufficient for synthesis for about 1 min. during normal exponential growth.

Incorporation of L-[Me-¹⁴C]methionine into Salmonella typhimurium. In their study of the chemical structure of flagellin from *S. typhimurium*, strain sw 1061, Ambler & Rees (1959) found the unusual amino acid, ϵ -N-methyllysine (NML), an amino acid not previously reported in any bacterial protein. This amino acid can be labelled by incubating cultures of *S. typhimurium* with either L-[U-¹⁴C]lysine or L-[Me-¹⁴C]methionine. The general pattern of labelling of *S. typhimurium* following incubation of the bacteria in a minimal medium containing L-[Me-¹⁴C]methionine is shown in Table 4. More than 95 % of the activity in the flagella was recovered as NML. The incorporation of the methyl group from L-[Me-¹⁴C]methionine into cellular fractions other than flagella was considered as a possible method for studying the initial stages and possible intermediates in the formation of flagella by *S. typhimurium*.

Table 4. *Distribution of ¹⁴C in cellular fractions of Salmonella typhimurium after incorporation of L-[Me-¹⁴C]methionine*

A suspension of *S. typhimurium* was incubated in the minimal medium supplemented with L-[Me-¹⁴C]methionine (0.05 μ C./ml., 10 μ g./ml.) After incubation for 4 hr. at 37° the bacteria were harvested by centrifugation and washed with distilled water. The suspension was divided into two parts, of which one was fractionated by a method based on that of Roberts *et al.* (1955), while the other was disrupted by ultrasonic vibration and fractionated by centrifugation. The radioactivity of the various fractions was determined.

Fraction	Radioactivity as % of the total
Chemical fractionation	
'Pool'	1.6
Ethanol soluble protein	4.3
Lipid	14
Nucleic acid	5
Acid insoluble residue	75.1
Physical fractionation	
10,000g pellet	22.2
100,000g pellet	28
Soluble fraction (includes 'pool')	49.8

Addition of L-[Me-¹⁴C]methionine to a suspension of *Salmonella typhimurium* incubated in minimal medium resulted in labelling the flagellar NML and of other cell constituents (Fig. 5). No samples were taken earlier than 2 min. after adding methionine, but extrapolation of the curve to zero time gave no indication of an appreciable lag before the rate of incorporation of the methyl group from methionine in the flagellar NML reached its maximum value. Incorporation in NML paralleled incorporation in the TCA-insoluble residue and gave a satisfactory measure of the rate of synthesis of flagellin. Mechanical removal of the flagella immediately before adding methionine did not affect the relative rates of labelling of flagella and residual protein.

Salmonella typhimurium has the same growth rate in a complex medium at 44° as at 37°, but at 44° the synthesis of flagella is inhibited and on continued incuba-

tion a non-flagellate culture is obtained. The rates of incorporation of L-[Me-¹⁴C]-methionine in the residual protein fractions by bacteria previously grown at 37° were the same at 44° as at 37° (Fig. 6a), but the rate of labelling of flagellar NML was markedly decreased after incubation for 5 min. at 44° as compared with the rate at 37° (Fig. 6b). The results obtained for the methyl labelling of flagellar ε-N-methyllysine were consistent with those obtained by using incorporation of other isotopically-labelled amino acids to follow flagellar synthesis, and this technique provided a satisfactory method for determining the rate of flagellar formation by *S. typhimurium*.

Fig. 3

Fig. 4

Fig. 5

Fig. 3. The effect of adding excess unlabelled leucine on the uptake of L-[U-¹⁴C]leucine by *Salmonella typhimurium*. L-[U-¹⁴C]leucine (final conc. 0.1 μ C./ml.) was added to a culture of *S. typhimurium* growing exponentially in the minimal medium supplemented with a mixture of 18 amino acids (50 μ g. of each/ml.). After 30 min. incubation at 37° the culture was divided into two and, to one portion, unlabelled leucine (final conc. 5 mg./ml.) was added. Samples were taken at intervals, the flagella were detached from the bacteria and precipitated serologically. The radioactivity of flagella (—●—●) and the acid-insoluble residue from the deflagellated bacteria (—○—○) was measured.

Fig. 4. Incorporation of L-[G-³H]leucine into exponentially growing *Salmonella typhimurium*. L-[G-³H]leucine (final conc. 1.6 μ C./ml.; specific activity 105 μ C./ μ M) was added to a culture of *S. typhimurium* growing exponentially in the minimal medium supplemented with a mixture of 18 amino acids (50 μ g. of each/ml.). Samples were taken at intervals and the flagella were detached mechanically from the bacteria and precipitated serologically. The incorporation of L-[G-³H]leucine into the flagella (—●—●) and the acid insoluble residue of the bacteria (—○—○) was determined.

Fig. 5. Incorporation of L-[Me-¹⁴C]methionine into *Salmonella typhimurium*. *S. typhimurium* growing exponentially in the minimal medium was harvested by centrifugation and resuspended in the same medium supplemented with L-[Me-¹⁴C]methionine (2.3 μ g./ml.; 0.1 μ C./ml.) and incubated at 37°. After sampling, the flagella were detached mechanically from the bacteria. The flagella and the acid-insoluble residue were hydrolysed with 6N-HCl at 105° for 16 hr. and after removal of the HCl, aliquots were electrophoresed at pH 10.6. The material corresponding to NML was eluted and its radioactivity determined. —●—●, NML in the flagella; —×—×, 'NML' in the acid-insoluble residue; —○—○, total activity in the acid insoluble residue.

Flagellar precursors in Salmonella typhimurium. Suspensions of *Salmonella typhimurium* were incubated in the minimal medium supplemented with L-[Me-¹⁴C]-methionine, and, after removal of the flagella by treatment in the Waring blender followed by centrifugation, were precipitated with TCA (final concentration 5%,

w/v). The residual protein fraction was hydrolysed with 6 N-HCl and the resulting hydrolysate electrophoresed in the presence of carrier NML. The areas corresponding to NML were cut out and the amino acid-ninhydrin complexes eluted and their radioactivity determined. The results of a typical experiment are shown in Fig. 5. The 'NML' of this fraction was labelled at approximately the same rate and to the same extent as the flagellar NML. The quantity of 'NML' detected in the deflagellated bacteria was much greater than expected from the values of 'intracellular flagellin' determined serologically. Similar results were obtained using L-[U- 14 C]lysine as the precursor of 'NML' (Table 5).

Fig. 6. Incorporation of L-[Me- 14 C]methionine into *Salmonella typhimurium*: (a) incorporation into the acid-insoluble material; (b) incorporation into flagellar NML. *S. typhimurium* growing exponentially at 37° was harvested and resuspended in the minimal medium supplemented with L-[Me- 14 C]methionine (2.8 μ g./ml.; 0.1 μ C./ml.) and incubated at either 37° or 44°. After sampling, the flagella were detached mechanically and separated by centrifugation from the bacteria. The acid-precipitated flagella were hydrolysed and the hydrolysate electrophoresed at pH 10.6. The material corresponding to NML was eluted and the radioactivity assayed. The incorporation of L-[Me- 14 C]methionine into the acid insoluble residue was also measured. -●-●-, Bacteria incubated at 37°; -○-○-, bacteria incubated at 44°.

The amount of L-[Me- 14 C]methionine present in the incubation medium was sufficient for incorporation to occur at the maximum rate for about 15–20 min. and subsequently the rate rapidly decreased. When incubation is continued for longer periods, any precursors labelled during the first 15 min. will be incorporated in flagella which will increase in radioactivity at the expense of the precursor fraction. Alternatively, an excess of unlabelled methionine can be added after the first 15 min. incubation. Addition of carrier-free L-[U- 14 C]lysine or L-[Me- 14 C]methionine to suspensions of *Salmonella typhimurium* incubated in minimal medium resulted in labelling of the flagellar NML and also 'NML' in both the ethanol-soluble protein and the residual protein fractions (Fig. 7a, b). In neither case was there any evidence for a decrease in the radioactivity of 'NML' in either the ethanol-soluble

Fig. 7a

Fig. 7b

Fig. 8

Fig. 7. Incorporation of L-[U-¹⁴C]lysine (a) and L-[Me-¹⁴C]methionine (b) into 'NML' by *Salmonella typhimurium*. *S. typhimurium* growing exponentially in the minimal medium was harvested and resuspended in the same medium supplemented with either L-[U-¹⁴C]lysine (1.7 μ g./ml.; 0.1 μ C./ml.) or L-[Me-¹⁴C]methionine (2.3 μ g./ml.; 0.1 μ C./ml.) and incubated at 37°. Samples were taken at intervals and the flagella removed mechanically. The deflagellated bacteria were fractionated into an ethanol-soluble and a residual protein fraction. These fractions were hydrolysed and the hydrolysates electrophoresed at pH 10.6 with an NML marker. The NML-ninhydrin complexes were eluted and the radioactivity determined. -●-●-, Flagella; -x-x-, ethanol soluble protein; -○-○-, residual protein.

Fig. 8. The effect of addition of unlabelled methionine on the distribution of ¹⁴C in cellular fractions of *Salmonella typhimurium* previously labelled with L-[Me-¹⁴C]methionine. *S. typhimurium* growing exponentially in the minimal medium was harvested and resuspended in the same medium supplemented with L-[Me-¹⁴C]methionine and incubated at 37°. After 15 min. incubation at 37°, unlabelled methionine was added (final conc. 100 μ g./ml.). Samples were taken at intervals, the flagella removed mechanically and the deflagellated bacteria disrupted ultrasonically. The disintegrated bacteria were fractionated by centrifugation into a low speed pellet (P₁), a high speed pellet (P₂), and a supernatant fraction (S). The proteins from these fractions were hydrolysed and the resulting amino acids separated electrophoretically. The NML-ninhydrin complexes were eluted and their radioactivity determined. -●-●-, Flagellar protein; -□-□-, low-speed pellet; -x-x-, high-speed pellet; -○-○-, supernatant fraction.

Table 5. The distribution of ¹⁴C in cellular fractions of *Salmonella typhimurium* after the incorporation of L-[U-¹⁴C]lysine or L-[Me-¹⁴C]methionine

Suspensions of *S. typhimurium* were incubated in the minimal medium supplemented with either L-[U-¹⁴C]lysine (0.1 μ C./ml.; 1.7 μ g./ml.) or L-[Me-¹⁴C]methionine (0.1 μ C./ml.; 2.3 μ g./ml.). After 15 min. incubation, the bacteria were harvested by centrifugation, the flagella removed mechanically and the deflagellated bacteria disrupted ultrasonically. The disintegrated bacteria were fractionated by centrifugation at 10,000g for 15 min. and 100,000g for 120 min. into a low speed pellet (P₁), a high-speed pellet (P₂) and a supernatant fraction (S). The acid-insoluble protein in these fractions was hydrolysed with 6 N-HCl and, after electrophoresis of the hydrolysate, the radioactivity of the 'NML' and the lysine was determined.

Amino acid	Cellular fraction							
	Flagella		P ₁		P ₂		S	
	NML	Lys.	NML	Lys.	NML	Lys.	NML	Lys.
	Radioactivity, counts/min./mg. dry wt bacteria							
L-[U- ¹⁴ C]lysine	370	570	36	670	88	6450	160	11640
L-[Me- ¹⁴ C]methionine	396	.	28	.	97	.	150	.

protein or the residual protein fractions corresponding to an increase in the flagellar NML. It is unlikely therefore that either fraction contains an appreciable amount of protein acting as a precursor for flagella.

The above chemical fractionation is arbitrary and a more satisfactory procedure was then adopted, based on the centrifugal separation of cell constituents after ultrasonic disintegration. The distribution of L-[*Me*-¹⁴C]methionine and L-[*U*-¹⁴C]-lysine in the 'NML' and lysine of the various cell fractions of *Salmonella typhimurium* after incubation in the minimal medium supplemented with the amino acid is shown in Table 5. Essentially the same pattern of labelling occurred in both cases and 'NML' was detected in all fractions. Addition of excess of unlabelled amino acid after 15 min. incubation in the presence of L-[*Me*-¹⁴C]methionine caused a decrease in the labelling of the 'NML' in the ribosome fraction equivalent to the increase in the labelling of 'NML' of the soluble protein fraction, but there was no corresponding increase in the radioactivity of flagellar NML (Fig. 8).

Preliminary electrophoretic separation of the proteins present in the soluble fraction indicated that the 'NML' was not confined to one particular protein but was distributed throughout many, if not all, the proteins present in this fraction.

DISCUSSION

The methods for flagellar assay described in this paper involve the incorporation of a radioactive amino acid followed by measurement of the radioactivity of the isolated flagella after separating them from the bacteria. It is impossible to remove flagella completely from bacteria but, by standardizing the experimental conditions, the number of flagellar 'stumps' remaining on the bacteria should be small and reasonably constant. Complete separation of the flagella from the deflagellated bacteria is difficult to achieve except by centrifugation at high speeds and a number of viable bacteria will be present in the supernatant fluid after centrifugation. An allowance must therefore be made for possible errors arising from these bacteria. Precipitation of flagella by adding TCA gives a measure of the radioactivity in both flagella and contaminating bacteria, and up to 5% of the radioactivity in the precipitate may be due to the presence of bacteria. The presence of short stumps of flagella on bacteria can result in their precipitation by anti-flagellar antiserum and also bacteria may be trapped in the antigen-antibody complex, so increasing the potential error of the assay.

The finding by Ambler & Rees (1959) that NML is present in the flagellin of *Salmonella typhimurium* strain sw1061, but not in appreciable amounts in its general cell protein suggested a more specific method of flagellar assay. NML was isotopically labelled with either L-[*U*-¹⁴C]lysine or L-[*Me*-¹⁴C]methionine and, after hydrolysis of the detached flagella, NML could be separated electrophoretically and its radioactivity determined. The percentage of radioactivity in NML due to other substances present in the precipitate is lower than the possible contamination in the other methods of flagellar assay. This method is now used routinely for studying the formation of flagella by *S. typhimurium*. The subsequent finding that the incorporation of either L-[*U*-¹⁴C]lysine or L-[*Me*-¹⁴C]methionine by *S. typhimurium* led to labelling of a compound present in the protein fraction of deflagellated bacteria which behaved electrophoretically as NML, did not invalidate the

method, since even the maximum contamination due to bacteria in the flagellar suspension was negligible.

The relative rates of amino acid incorporation into the flagella and the general cell protein agree very well with the findings of Stocker & Campbell (1959) and Quadling & Stocker (1962), with one exception. The incorporation of methionine in flagella of *Salmonella typhimurium* incubated at 44° decreased rapidly although incorporation into the general cell protein continued at the same rate as in the control culture, whereas Quadling & Stocker (1962) found that synthesis of flagella continued for about one generation time at 44°. This discrepancy is probably due to the fact that these authors used a complex medium, whereas the incorporation of L-[Me-¹⁴C]-methionine was studied in a glucose + ammonium salts medium in which *S. typhimurium* cannot grow at 44°.

The fact that flagellin is detectable in the soluble protein fraction of both *Proteus vulgaris* (Weinstein *et al.* 1960) and *Salmonella typhimurium* raised the question of possible soluble protein intermediates in the formation of functional flagella by these organisms. There are, of course, other possible explanations for the presence of flagellin within the cell: for example, the flagellin could merely be a by-product of the synthetic pathway, or might arise from breakdown of flagella during disruption of the bacteria. However, the finding that flagella may be degraded during cell breakage does not exclude the possibility that a fraction of the 'intracellular flagellin' is acting as an intermediate in the formation of the flagella.

The absence of a lag before the appearance of either L-[G-¹³H]leucine or L-[Me-¹⁴C]-methionine in the flagella of *Salmonella typhimurium* after adding the amino acid to the culture suggests that any pool of intermediates is small. A lag of about 1 min. would be expected before the rate of incorporation reached its maximum value if all the flagellin in the soluble protein fraction was to act as an intermediate. No lag of this type was ever detected in the incorporation experiments.

The labelling of the flagellar NML by L-[Me-¹⁴C]methionine provided a satisfactory measure of the rate of flagellar formation by *Salmonella typhimurium* and it was hoped that tracing NML in other cellular fractions might provide evidence either for or against flagellar precursors. The quantity of 'NML' detected in deflagellated bacteria after incorporation of either L-[U-¹⁴C]lysine or L-[Me-¹⁴C]-methionine was considerably greater than the value expected from the serological assay of 'intracellular flagellin'. This intracellular 'NML' was not isolated and chemically characterized, but the isotopic labelling and its electrophoretic behaviour were identical with those of flagellar NML. The possibility that the 'NML' containing material present within the bacterium, or some part of it, was acting as a flagellar precursor was studied by using pulse-labelling and chasing with the unlabelled amino acid. In no case was it possible to show that the radioactivity of the flagellar NML increased concomitantly with a decrease in the radioactivity of 'NML' in any fraction of the bacteria. Preliminary electrophoretic separation of proteins from the soluble fraction of disrupted bacteria indicated that 'NML' may be present in trace amounts in a number of bacterial proteins and is not confined to flagellin. Stocker, McDonough & Ambler (1961) suggested that the methylation of lysine may occur after its incorporation in the polypeptide chain of flagellin, in which case the small amount of 'NML' detected in other cellular proteins could be due to a lack of absolute specificity of the methylating system for flagellin. The

failure to find any soluble protein intermediates in the formation of flagella does not preclude the possibility that such precursors occur in extremely small amounts. The complete absence of soluble intermediates would imply that the ribosomes synthesizing flagellin molecules are located at the site of aggregation of the individual molecules to form the functional flagellum.

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