Flagellar Synthesis in *Salmonella typhimurium*: Factors Affecting the Formation of the Flagellar ϵ -N-Methyllysine

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SUMMARY

In growing cultures of *Salmonella typhimurium*, flagellar ϵ -N-methyllysine (NML) can be isotopically labelled with either L-[Me- 14 C]methionine or L-[U- 14 C]lysine. This isotopic labelling of flagellar NML was unaffected by the addition of DL-NML to the incubation medium. The methionine analogues, D-methionine and DL-ethionine, competitively inhibited the incorporation of the methyl group from methionine into flagellar NML, but had no effect on the incorporation of L-[U- 14 C]lysine. Although it is probable that methylation of lysine occurs after it has been incorporated into the flagellin molecule, it has not been possible by using antibiotics and purine and pyrimidine analogues to differentiate between flagellar synthesis and the formation of flagellar NML. Attempts to obtain a cell-free preparation capable of synthesizing NML have so far been unsuccessful.

INTRODUCTION

The presence of ϵ -N-methyllysine (NML) in flagellar protein (flagellin) of *Salmonella typhimurium* strain sw 1061 was first reported by Ambler & Rees (1959), and since then this amino acid has been found in flagellins from other serotypes of *S. typhimurium* (Stocker, McDonough & Ambler, 1961) and in calf thymus nuclear histones (Murray, 1964). The methyl donor for the synthesis of NML in both bacterial flagella (Kerridge, 1963) and nuclear histone (Murray, 1964; Allfrey, Faulkner, & Mirsky, 1964) is methionine, and it was first suggested by Stocker *et al.* (1961) that this methylation might occur after the incorporation of lysine into the protein molecule. More recently, Allfrey *et al.* (1964) have found that, although puromycin inhibits the synthesis of protein by isolated calf thymus nuclei, this antibiotic has little or no effect on the methylation of the histone lysine.

The incorporation of labelled precursors into NML of the flagellar protein by *Salmonella typhimurium* was used by Kerridge (1963) as a measure of flagellar synthesis, and further studies on the factors affecting the synthesis of flagellar NML are reported in this paper. A preliminary account of some of the results has already been given (Kerridge, 1962).

METHODS

Organisms and growth media. *Salmonella typhimurium* strain sw 1061, obtained from Dr Iino (National Institute of Genetics, Misima, Shizuoko-ken, Japan), was used for the majority of the experiments reported in this paper. Other strains of

S. typhimurium used were sw 1153 (paralysed), sw 1154 (non-flagellate) and sl 871 (*his*⁻, NML⁻): these were obtained from Professor B. A. D. Stocker, The Lister Institute, London. The cultures were maintained on Dorset egg slopes at room temperature. For the preparation of washed suspensions the bacteria were grown in an ammonium salts medium (DO) supplemented with 0.2% (w/v) glucose (Kerridge, 1959). After 16 hr growth at 37° the culture was diluted with 20 vol. of DO medium + 0.2% (w/v) glucose and the incubation continued for a further 4 hr. The bacteria were harvested by centrifugation and resuspended in DO at a density of 5 mg. dry wt./ml.

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

Incorporation of ¹⁴C-labelled amino acids into Salmonella typhimurium. Suspensions of *Salmonella typhimurium* (0.5 mg. dry wt./ml.) were incubated statically at 37° in DO + 0.2% (w/v) glucose. L-[Me-¹⁴C]methionine (specific activity 6.6 mC./mmole) or L-[U-¹⁴C]lysine (specific activity 8.3 mC./mmole) was added to give a final concentration of 0.1 µC./ml. and the medium was supplemented with other amino acids or inhibitors as required. 5 ml. samples were taken at intervals and pipetted into centrifuge tubes containing 5 ml. of an aqueous solution of the unlabelled amino acid (100 µg./ml.). After cooling rapidly to 0°, the bacteria were harvested by centrifugation at 1300g for 20 min. in the cold, and resuspended in 2.5 ml. cold distilled water. The flagella were detached mechanically by treatment in a M.S.E. microhomogenizer and separated from the bacteria by centrifugation at 1300g for 20 min. The fractionation of the bacteria and the determination of the isotope incorporation into the various cellular fractions were done by the methods described by Kerridge (1963). The radioactivity of the flagellar NML was determined using a thin end-window gas flow counter (Nuclear Chicago Corp., 333 East Howard Avenue, Des Plaines, Illinois, U.S.A.), the efficiency of which is approximately three times that of the Panax type D 657 Scaler (Panax Equipment Ltd., Mitcham, Surrey) and associated mica end-window Gieger-Muller tube used for the assay of radioactivity in the other cellular fractions.

RESULTS

The effects of methionine, lysine and NML on the incorporation of ¹⁴C-labelled amino acids by Salmonella typhimurium. The rate of incorporation of an isotopically labelled amino acid into flagella by *Salmonella typhimurium* under normal conditions of growth parallels the rate of incorporation into other cellular fractions and provides a satisfactory measure of flagellar formation. A more specific method of studying flagellar synthesis in *S. typhimurium* is to follow the incorporation of ¹⁴C-labelled precursors into flagellar NML, and studies have been made on the factors affecting the labelling of flagellar NML in an attempt to differentiate between protein synthesis and NML formation.

The incorporation of L-[Me-¹⁴C]methionine into general cell protein was slightly stimulated by adding lysine to the incubation medium, but there was a more marked increase in the incorporation of ¹⁴C into the flagellar NML (Table 1). This was

detectable at fairly low concentrations of lysine and tended towards a plateau value as the concentration of lysine was increased.

The incorporation of ^{14}C from L-[Me- ^{14}C]methionine into lipid, nucleic acid, and protein by *Salmonella typhimurium* was unaffected by the addition of NML to the incubation medium, and there was no reduction in the methyl labelling of the

Table 1. *The effect of lysine on the incorporation of L-[Me- ^{14}C]methionine into Salmonella typhimurium strain sw 1061*

Suspensions of *S. typhimurium* (0.5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0.2%, w/v) and L-[Me- ^{14}C]methionine (0.1 μC ./ml.; specific activity 6.6 mC./mmole). L-Lysine was added as required. After 15 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined. *a* and *b* represent separate experiments.

	L-Lysine added (μg ./ml.)				
	0	10	50	100	200
	Radioactivity (counts/min./mg. bacteria)				
Expt. <i>a</i>					
Protein	3500	—	4200	4000	4540
Flagellar NML	52	124	164	148	150
Expt. <i>b</i>					
Protein	4100	4060	4900	4330	4540
Flagellar NML	230	260	310	370	320

Table 2. *The effect of NML on the incorporation of L-[Me- ^{14}C]methionine into Salmonella typhimurium strain sw 1061*

Suspensions of *S. typhimurium* (0.5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0.2%, w/v) and L-[Me- ^{14}C]methionine (0.1 μC ./ml.; specific activity, 6.6 mC./mmole). DL-NML was added as required. After 10 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined.

	DL-NML added (μg ./ml.)			
	0	10	30	100
	Radioactivity (counts/min./mg. bacteria)			
Cell fraction				
Lipid	1870	1870	2050	2090
Nucleic acid	300	264	330	350
Protein (acid insoluble residue)	7280	7120	6750	7800
'NML'				
Flagella	276	260	314	342
Protein (acid-insoluble residue)	330	340	332	—

flagellar NML even when the molar ratio of NML/methionine was approximately 40/1 (Table 2). In the presence of exogenous NML there appeared to be no reduction in the incorporation of ^{14}C from L-[Me- ^{14}C]methionine into cellular 'NML'.

L-[U- ^{14}C]lysine is also incorporated into flagellin by *Salmonella typhimurium* and the label is found in both the NML and lysine residues, but it is not such a satisfactory precursor as L-[Me- ^{14}C]methionine for studying formation of bacterial flagella (Kerridge, 1963). Addition of carrier-free L-[U- ^{14}C]lysine to a suspension of *S. typhimurium* in the glucose ammonium salts medium resulted in an immediate

linear incorporation of the amino acid into the bacteria. After 15 min. incubation, the rate of lysine incorporation into the flagellar NML fell rapidly and after 30 min. was practically zero, but the radioactivity of the lysine in the flagellar fraction continued to rise for at least an hour (Fig. 1). This increase may have been due to

Table 3. *The effect of methionine on the incorporation of L-[U-¹⁴C]lysine into Salmonella typhimurium strain sw 1061*

Suspensions of *S. typhimurium* (0.5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0.2 %, w/v) and L-[U-¹⁴C]lysine (0.1 µC./ml.; specific activity 8.3 mC./mmole). L-Methionine was added as required. After 15 min. the bacteria were harvested by centrifugation, fractionated and the radioactivity of the various fractions determined.

	Methionine (µg./ml.)				
	0	10	50	100	200
	Radioactivity (counts/min./mg. bacteria)				
Protein fraction	17,900	13,700	19,000	19,100	17,600
Flagella					
(a) NML	780	870	880	880	880
(b) Lysine	1,585	1,480	1,540	1,740	1,430

Table 4. *The effect of NML on the incorporation of L-[U-¹⁴C]lysine into Salmonella typhimurium strain sw 1061*

Suspensions of *S. typhimurium* (0.5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0.2 %, w/v) and L-[U-¹⁴C]lysine (0.1 µC./ml.; specific activity, 8.3 mC./mmole). DL-NML was added as required. After 10 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined.

	DL-NML (µg./ml.)			
	0	10	30	100
	Radioactivity (counts/min./mg. bacteria)			
Cell fraction				
Lipid	27	20	24	10
Nucleic acid	515	625	520	350
Protein (acid-insoluble residue)	12,300	13,950	13,700	12,200
'NML'				
Flagella	140	144	148	132
Protein (acid-insoluble residue)	212	264	140	140

the presence of proteins other than flagellin in this fraction, probably 'released' from the bacterial cells. The presence of methionine in the medium had little or no effect on the incorporation of L-[U-¹⁴C]lysine into flagellar NML and lysine (Table 3). The incorporation of L-[U-¹⁴C]lysine into flagellar NML and lysine was unaffected by the addition of NML to the incubation medium, but there was some reduction in the isotopic labelling of the cellular 'NML' (Table 4).

The incorporation of precursors into NML by Salmonella strains other than sw 1061
NML is not a constituent of all *Salmonella* flagellins, and methionine incorporation has been studied in a number of strains of *Salmonella typhimurium* which either do not have this amino acid in their flagella or are non-motile as a result of 'paralysis'

or absence of flagella. The paralysed strain sw 1153 behaved almost identically to the wild-type organism (strain sw 1061) and the ^{14}C -labelled methyl group from methionine was incorporated into the flagellar NML after a short lag (Fig. 2). The isotope was also incorporated into a constituent of the residual protein fraction which behaved electrophoretically like NML. *S. typhimurium* strain SL 871, a strain having no NML in its flagellin, and the flagella-less strain sw 1154 behaved exactly as expected, and no radioactivity was detected in the NML region after electrophoresis of an acid hydrolysate of the 'flagellar fractions'. These results demonstrated satisfactorily that other cellular constituents did not contribute significantly to the radioactivity of the NML in the flagellar fraction. In both SL 871 and sw 1154 some radioactivity was detected in the NML region after electrophoresis of an acid hydrolysate of the residual protein.

Fig. 1.

Fig. 2.

Fig. 1. The incorporation of L-[U- ^{14}C]lysine into flagellar protein by *Salmonella typhimurium*, strain sw 1061. A suspension of *S. typhimurium* (0.5 mg. dry wt./ml.) was incubated in DO medium containing glucose (0.2%, w/v) and L-[U- ^{14}C]lysine (0.1 $\mu\text{C.}$ /ml.; specific activity 8.3 $\mu\text{mC.}$ /mmole). Samples were taken at intervals and the radioactivity of the flagellar NML and lysine determined. ●—●, NML; ○—○, lysine.

Fig. 2. The incorporation of L-[Me- ^{14}C]methionine into non-motile strains of *Salmonella typhimurium*. Suspensions of strains sw 1153 (*mot*⁻) and sw 1154 (*fla*⁻) (0.5 mg. dry wt./ml.) were incubated in DO medium containing glucose (0.2%, w/v) and L-[Me- ^{14}C]methionine (0.1 $\mu\text{C.}$ /ml.; specific activity 6.6 mC./mmole). Samples were taken at intervals, the bacteria harvested by centrifugation, fractionated, and the radioactivity of the various cellular fractions determined. ●—●, Flagellar NML, sw 1153; ×—×, 'flagellar NML', sw 1154. The figures in parentheses give the radioactivity of the 'NML' in the residual protein fraction at the time indicated.

The effects of methionine analogues on the incorporation of L-[Me- ^{14}C]methionine and L-[U- ^{14}C]lysine into Salmonella typhimurium. The methionine analogues, D-methionine, methionine sulphone, methionine sulphoxide and ethionine, did not prevent bacterial growth in defined media or the regeneration of flagella by previously deflagellated bacteria. However, these analogues decreased the incorporation of isotope from L-[Me- ^{14}C]methionine into *S. typhimurium* (Figs. 3, 4) and there was

no obvious differential effect on the methylation of the flagellar lysine and the incorporation of methionine into the residual protein fraction. D-Methionine and DL-ethionine were shown to inhibit competitively both the incorporation of methionine into the residual protein fraction and the methylation of the flagellar lysine.

D-Methionine and DL-ethionine had no effect on the incorporation of L-[U- 14 C]-lysine into either the residual protein fraction or into flagellar NML (Table 5) so, although these analogues prevent the incorporation of the methyl group from exogenous methionine into the flagellar NML, they do not prevent the synthesis of this compound by *Salmonella typhimurium*.

The effects of antibiotics on the synthesis of flagellar NML. If methylation of lysine occurs after the amino acid has been incorporated into the flagellin molecule, then antibiotics known to inhibit protein synthesis might have a differential effect on the methylation of lysine and on the incorporation of methionine into cell protein.

Fig. 3a.

Fig. 3b.

Fig. 4.

Fig. 3. The effect of methionine analogues on the incorporation of L-[Me- 14 C]methionine into *Salmonella typhimurium*. Suspensions of *S. typhimurium* (0.5 mg. dry wt./ml.) were incubated at 37° in DO medium containing glucose (0.2%, w/v) and L-[Me- 14 C]methionine. The methionine analogues were added as required. After 15 min. the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined. (a) Incorporation into the acid-insoluble material (protein). (b) Incorporation into flagellar NML. x—x, D-methionine; ●—●, DL-methionine sulphone; O—O, DL-methionine sulphoxide.

Fig. 4. The effect of DL-ethionine on the incorporation of L-[Me- 14 C]methionine into *Salmonella typhimurium*. Suspensions of *S. typhimurium* strain sw 1061 were incubated in DO medium containing glucose (0.2%, w/v) and L-[Me- 14 C]methionine (0.1 μ C./ml.; specific activity 6.6 mC./mmole). Ethionine was added as required. After 15 min. the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined. O—O, flagellar NML; ●—●, residual protein fraction.

Allfrey *et al.* (1964) were able to demonstrate such an effect with puromycin on the methylation of lysine residues in histones and the synthesis of protein by isolated calf thymus nuclei. Similar experiments were therefore carried out with *Salmonella typhimurium*. At the minimum growth inhibitory concentrations the antibiotics chloramphenicol, terramycin, and puromycin inhibited the regeneration of flagella by deflagellated bacteria (Table 6), and so smaller concentrations were used in attempts to show a differential effect on the methylation of lysine and the synthesis of protein. The effects of the antibiotics on the incorporation of 14 C from L-[Me- 14 C]-methionine into nucleic acid and protein were in good agreement with the results of other workers (see Gale, 1963). At all concentrations of the antibiotics tested,

the inhibition of methyl labelling of flagellar NML paralleled the inhibition of ^{14}C -incorporation into the residual protein fractions, and no differential effects were detected (Table 7). The incorporation of methyl C into the material present in the residual protein fraction which behaved electrophoretically as NML was

Table 5. *The effects of DL-ethionine and D-methionine on the incorporation of L-[U- ^{14}C]lysine into Salmonella typhimurium strain sw 1061*

Suspensions of *S. typhimurium* (0.5 mg./ml.) were incubated statically at 37° in DO medium containing glucose (0.2 %, w/v) and L-[U- ^{14}C]lysine (0.1 μC ./ml.; specific activity 8.3 mC./mmole). DL-Ethionine or D-methionine was added as required. After 10 min. incubation at 37° the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined.

Cell fraction	Experiment 1		Experiment 2	
	Control	+ 100 μg . DL- ethionine/ ml.	Control	+ 1 mg. D- methionine/ ml.
Lipid	15	30	130	40
Nucleic acid	300	310	680	490
Protein (acid-insoluble residue)	12,900	13,300	22,500	22,600
'NML'				
Flagella	450	435	570	660
Acid-insoluble residue	530	280	370	405

Table 6. *The effects of inhibitors on the regeneration of flagella by Salmonella typhimurium strain sw 1061*

Suspensions of mechanically deflagellated bacteria (0.2 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0.2 %, w/v) and Difco vitamin-free casamino acids (0.2 %, w/v). The inhibitors were added as required. The regeneration was followed microscopically by motility determinations and flagella staining (Kerridge, 1959).

	Regeneration after 1 hr. incubation	
	Motility (%)	Flagellated bacteria (%)
Control	90	90
Puromycin, 10 μg ./ml.	70	70
Puromycin, 30 μg ./ml.	10	10
Puromycin, 100 μg ./ml.	1	Very few short stumps of flagella
Terramycin, 0.3 μg ./ml.	1	A few very short stumps
Terramycin, 1.0 μg ./ml.	1	A few very short stumps
Terramycin, 3 μg ./ml.	1	A few very short stumps
6-Thioguanine, 10 μg ./ml.	90	90
6-Thioguanine, 100 μg ./ml.	90	90

apparently not as sensitive to inhibition by chloramphenicol as the methyl labelling of the flagellar NML; for example, at 10 μg . chloramphenicol/ml. the methyl labelling of the flagellar NML was 5 % of the control value, whereas that of the residual protein 'NML' was 30 % and the incorporation of ^{14}C into the residual protein fraction was 8 %.

Dr R. J. Martinez, in an unpublished symposium, said that flagella synthesis in *Spirillum serpens* can occur in the presence of growth-inhibiting concentrations of chloramphenicol, suggesting that there may be an intermediary pool of

flagellin molecules in this organism. Previous results suggest that if such a pool of flagellin molecules occurs in *Salmonella typhimurium* it must be small (Kerridge, 1963). The addition of chloramphenicol to a culture of *S. typhimurium* incubated in the presence of L-[Me-¹⁴C]methionine immediately inhibited ¹⁴C incorporation into flagellar NML (Fig. 5). This and the earlier results support the idea that *S. typhimurium* unlike *S. serpens* does not have a large pool of flagellin molecules that can be used for flagellar synthesis.

Table 7. *The effects of antibiotics on the incorporation of L-[Me-¹⁴C]methionine into Salmonella typhimurium strain sw 1061*

Suspensions of *S. typhimurium* (0.5 mg./ml.) were incubated in DO medium containing glucose (0.2 %, w/v) and L-[Me-¹⁴C]methionine (0.1 μ C./ml.; specific activity 6.6 mC./mmole). The antibiotics were added as required. After 10 min. at 37° the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined.

Fraction	Radioactivity (counts/min./mg. bacteria)			
	Chloramphenicol (μ g./ml.)			
	0	1	3	10
Lipid	1280	1180	1380	1320
Nucleic acid	380	350	310	280
Protein (acid-insoluble material)	7400	2670	1210	610
'NML'				
Flagella	310	60	19	4
Residual protein	150	—	—	46
Fraction	Puromycin (μ g./ml.)			
	0	10	30	100
	0	10	30	100
Lipid	720	760	720	680
Nucleic acid	320	380	310	300
Protein (acid-insoluble residue)	5180	6700	4900	2420
'NML'				
Flagella	190	170	67	22
Fraction	Terramycin (μ g./ml.)			
	0	0.08	0.1	0.3
	0	0.08	0.1	0.3
Lipid	1390	1240	1920	2350
Nucleic acid	330	325	293	240
Protein (acid-insoluble residue)	6200	3170	2030	1350
'NML'				
Flagella	93	30	21	24
Acid-insoluble residue	76	56	28	40

If the methylation of lysine in *Salmonella typhimurium* occurs before the amino acid is incorporated into the flagellin molecule it should be possible to detect NML in the low molecular weight fraction of the bacteria. So far this has not proved possible, and even under conditions where protein synthesis was inhibited by the addition of antibiotics, ¹⁴C-methyl labelled NML could not be detected in either the culture supernatant fluid or in the low molecular weight fraction of the bacteria.

Actinomycin D inhibits RNA synthesis *in vivo* in certain Gram-positive bacteria, but not in *Escherichia coli* (Kirk, 1960; Hurwitz, Furth, Malamy & Alexander, 1962), but it has recently been reported that pre-treatment of *E. coli* with 10⁻³ M

ethylenediaminetetraacetic acid (EDTA) at pH 8.0 renders the bacteria completely sensitive to the inhibitory action of actinomycin D (Leive, 1965). *Salmonella typhimurium* strain sw 1061 was unaffected by incubation in tris + HCl buffer (pH 8.0) containing EDTA (10^{-2} M) for 2 min., and the subsequent addition of 50 μ g. actinomycin D had no effect on the rates of incorporation of either L-[U- 14 C]leucine or [8- 14 C]adenine into the acid-insoluble material. Actinomycin could therefore not be used to study the stability of the messenger-RNA involved in the synthesis of flagella by *S. typhimurium* strain sw 1061.

Fig. 5.

Fig. 6.

Fig. 5. The effect of chloramphenicol on methyl labelling of flagellar NML. A suspension of *Salmonella typhimurium* strain sw 1061 (0.5 mg. dry wt./ml.) was incubated in DO medium containing glucose (0.2 %, w/v) and L-[Me- 14 C]methionine (0.1 μ C./ml.; specific activity, 6.6 mC./mmole). After 10 min. incubation at 37° samples were pipetted into conical flasks containing chloramphenicol and incubation continued. The cultures were sampled at intervals and the radioactivity of the flagellar NML determined. ○—○, Control; ●—●, with 1 μ g./ml. chloramphenicol; ×—×, with 10 μ g./ml. chloramphenicol.

Fig. 6. The effect of phenol on the incorporation of L-[Me- 14 C]methionine into *Salmonella typhimurium* strain sw 1061. Suspensions containing 0.5 mg. dry wt./ml. were incubated at 37° in DO medium supplemented with glucose (0.2 %, w/v) and L-[Me- 14 C]methionine. Phenol was added as required. After 15 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined. ●—●, Residual protein; ×—×, residual protein 'NML'; ○—○, flagellar NML.

The effects of inhibitors other than antibiotics on the synthesis of flagellar NML. For many years the addition of phenol (0.1 %, w/v) to solid media has been used to obtain non-flagellated bacteria (see, for example, Kramer & Koch, 1931), and recently Iino & Mitani (1962) suggested that phenol inhibits the multiplication of the 'flagella-forming apparatus' but not the formation of flagellar protein. Experi-

ments have been carried out to see if there is a differential effect on the synthesis of flagellar NML and growth in *Salmonella typhimurium* (Fig. 6). There was no obvious differential inhibition by phenol of the incorporation of ^{14}C into flagellar NML and into general cell protein during incubation for 15 min. This result is quite consistent with those of Iino & Mitani, and would suggest that phenol does not immediately inhibit the functioning of the existing 'flagella-forming apparatus'. There is no evidence as to the nature of this apparatus, which could be an integral part of the 'cell wall, membrane' complex.

Table 8. *The effects of analogues of purines and pyrimidines on the incorporation of L-[Me- ^{14}C]methionine into Salmonella typhimurium strain sw 1061*

Suspensions containing 0.5 mg. dry wt./ml. were incubated statically at 37° in DO medium containing glucose (0.2 %, w/v) and L-[Me- ^{14}C]methionine (0.1 $\mu\text{C.}/\text{ml.}$; specific activity 6.6 mC./mmole). The analogues were added as required. After 10 min. the bacteria were harvested by centrifugation, and the radioactivity of the various fractions determined.

	Radioactivity (counts/min./mg. dry wt. bacteria)	
	Cell protein	Flagellar NML
Control	3960	145
Thiouracil (3×10^{-3} M)	2450	135
5-Fluorouracil (3.8×10^{-4} M)	3260	138
6-Azauracil (2.7×10^{-4} M)	3600	121
6-Azauracil (8.8×10^{-4} M)	2370	96
FDUR (10^{-3} M)	3660	130
8-Azaguanine (10^{-3} M)	3240	132
6-Thioguanine (6×10^{-4} M)	3800	155

FDUR = fluorodeoxyuridine.

A number of purine and pyrimidine analogues are known not to affect the synthesis of existing flagella by *Salmonella typhimurium*, but certain of them, for example 8-azaguanine, apparently prevent the formation of new flagella (Kerridge, 1963). However, Mandel, Latimer & Riis (1965) reported that growth of *Bacillus cereus* in the presence of 6-thioguanine resulted in the production of non-flagellated bacteria, and they suggested that the analogue preferentially inhibited the formation of flagella. In *S. typhimurium* strain sw 1061 6-thioguanine at 0.6×10^{-3} M did not prevent the regeneration of functional flagella after mechanical removal (Table 6), and growth in the presence of the analogue did not produce a predominantly non-flagellated culture. Thioguanine and other analogues were tested for their effects on the incorporation of L-[Me- ^{14}C]methionine into *S. typhimurium* (Table 8). Even at the highest concentrations used there was incomplete inhibition of incorporation of ^{14}C into the residual protein fraction, and none of the analogues showed any marked differential effect on the methyl labelling of flagellar NML and the incorporation of ^{14}C into the residual protein fraction during the incubation for 10 min.

Synthesis of NML by cell-free extracts of Salmonella typhimurium. Suspensions were disrupted using either the ultrasonic disintegrator or the French pressure cell, and the broken bacteria fractionated by centrifugation (Kerridge, 1963). S-Adenosyl

methionine or L-[Me-¹⁴C]methionine with ATP and an energy generating system were used as the methyl donors and lysine or flagellin from *S. typhimurium* strain SL 871 (NML⁻) were used as methyl acceptors, but in no case was it possible to show any synthesis of NML.

DISCUSSION

These results confirm the earlier finding that in *Salmonella typhimurium* the incorporation of the methyl group from L-[Me-¹⁴C]methionine into flagellar NML is a satisfactory method of following the synthesis of flagella in this organism. The increased incorporation of ¹⁴C into flagellar NML following the addition of lysine to the incubation medium was a little surprising. Exogenously added lysine is unlikely to alter the NML content of the flagellin and one possible explanation is that the cells can synthesize more flagellin in the presence of lysine. The number of flagella per bacterium does depend to a large extent on the complexity of the growth medium (Stocker & Campbell, 1959; Kerridge, 1959) and in a minimal medium the rate of lysine synthesis, although sufficient to support formation of essential proteins, may possibly be limiting for the formation of the non-essential flagella. Exogenous lysine would overcome this limitation on flagella synthesis.

The methylation of 'flagellar' lysine by exogenously added methionine was not separated from the synthesis of flagellin molecules except by the addition of methionine analogues to the incubation medium. These did not themselves inhibit the synthesis of flagellar NML, since L-[U-¹⁴C]lysine was still incorporated into flagellar NML in their presence. If the methylation of lysine in flagellin is specific and the ratio lysine:NML is constant for this protein, it is not surprising that inhibitors of protein synthesis have a similar effect on the incorporation of Me-¹⁴C into flagellar NML. The effect on the 'NML' present in the residual protein fraction was not so clear-cut, and in the presence of inhibitors of protein synthesis this methylation was perhaps not inhibited to the same extent as the methylation of flagellar NML, a result in agreement with the findings of Allfrey *et al.* (1964) for the methylation of calf thymus histones. However, the reduction in labelling of the residual protein 'NML' by exogenous NML conflicts with this interpretation, and further studies are needed on the localization and formation of this material.

The purine and pyrimidine analogues tested had no obvious effects on the methylation of lysine by *Salmonella typhimurium*, but, as the incorporation of ¹⁴C into flagellar NML was followed for only 15 min., any long-term effects which might result from the incorporation of these compounds into nucleic acid would not be detected. The absence of a differential effect of thioguanine on flagella formation by *S. typhimurium*, similar to that found in *Bacillus cereus* by Mandel *et al.* (1965), might well be due to the different bacteria studied.

The stage in the synthesis of flagella at which methylation of lysine occurs has still to be determined. The failure to detect NML in the low molecular weight fraction of *Salmonella typhimurium* and the absence of any effect of exogenously added NML on the incorporation of either L-[Me-¹⁴C]methionine or L-[U-¹⁴C]lysine into flagellar NML are further evidence in favour of methylation occurring after the lysine has been incorporated into flagellin. Formation of flagellar NML does not occur in the presence of agents known to inhibit protein synthesis in *S. typhimurium*, but this cannot be considered as evidence for NML being incorporated as

such into the flagellin molecule. Until a satisfactory system for the cell-free methylation of lysine is developed, our information on the stage in the formation of flagella at which lysine is methylated must of necessity be circumstantial. Cell-free methylating systems have been developed for the methylation of individual amino acids (Blumenstein & Williams, 1963), polynucleotides (Fleissner & Borek, 1963) and nuclear histone (Allfrey *et al.* 1964), and unless the structural integrity of the cell is necessary for the synthesis of flagellar NML by *S. typhimurium* it should be possible to obtain an enzyme system from this organism capable of methylating lysine.

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