Methylation of the Flagellin of
Salmonella typhimurium

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The methylation of endogenous proteins by *Salmonella typhimurium* SL 870 was investigated in cell-free extracts by using *S*-adenosylmethionine as methyl donor. Several lines of evidence are presented which suggest that one of the methylated products is the protein flagellin. Mutant strains of SL 870 (*nml⁻fla⁺* and *nml⁺fla⁻*) were also found to synthesize epsilon-*N*-methyl-lysine. It is proposed that *S. typhimurium* possesses 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.

Enzymatic mechanisms exist for the alteration of monomers within preformed macromolecules. The modifications may have profound biological effects. For example, a change in the methylation of phage lambda deoxyribonucleic acid (DNA) altered its ability to undergo host-induced modifications (4); hydroxymethyl cytosine, whether glucosylated or not, protects progeny phage T4 against nuclease activity that degrades the cytosine-containing DNA of the host (21). The methylation of the epsilon amino group of certain lysine residues in histones may influence the binding of histones to DNA (7).

The occurrence of epsilon-*N*-methyl-lysine (NML) in *Salmonella* flagellin (the monomeric unit of flagella) was reported by Ambler and Rees (2). Other natural sources of this amino acid are certain histone fractions isolated from various mammalian tissues (14), ribosomes of *Blastocladiella emersonii* (9), and the flagella of *Spirillum serpens* (10).

Stocker, McDonough, and Ambler (20) found that the gene determining the presence or absence of NML in *Salmonella* flagellin maps separately from the structural gene for flagellin. They suggested that it codes for an enzyme which specifically methylates the epsilon amino groups of certain lysine residues in flagellin.

Kerridge (11) demonstrated that NML in *S. typhimurium* flagella is derived from lysine and the methyl group of methionine and that NML was not incorporated as such into the growing polypeptide chain. Kerridge, however, was unable to demonstrate NML synthesis by cell-free extracts with ¹⁴CH₃-*S*-adenosylmethionine (SAM)

as methyl donor. Comb et al. (9) reported biosynthesis of NML by washed ribosomes from *S. typhimurium*. Since they did not identify the methylated protein, it is not known whether ribosomal protein, ribosome-bound flagellin, or some other protein was methylated.

This report describes a cell-free system isolated from *S. typhimurium* which methylates protein. Evidence is presented that flagellin is methylated by these extracts, and that methylation does not require protein synthesis. Further, data are presented demonstrating that proteins other than flagellin are methylated by enzyme(s) different from the one responsible for flagellin methylation.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* strains SL 870, SL 871, and SL 4077 were provided by Bruce Stocker. SL 870 requires histidine for growth, is *fla⁺ nml⁺*, and is monophasic (*ahl⁻*). SL 871, obtained as a spontaneous mutant from SL 870 (2), is *fla⁺ nml⁻ his⁻*. SL 4077 is a nonflagellate (*fla⁻*) mutant of SL 870 obtained by selection with chi phage which attacks only motile cells.

Growth of bacteria. For the preparation of flagella and cell extracts, bacteria were grown in YA broth, which contains (per liter of distilled water): 8 g of Nutrient Broth (Difco) and 3 g of Yeast Extract (Difco), *p*H 7.0.

For some experiments, cells were grown on Spizizen's minimal medium containing (per liter of distilled water): K₂HPO₄, 14 g; KH₂PO₄, 6 g; (NH₄)₂SO₄, 2 g; sodium citrate, 1 g; MgSO₄·7H₂O, 0.2 g; glucose, 5 g; and 10.0 ml of 1 × 10⁻² M histidine.

Highly motile cells for inoculating growth flasks were selected by growing bacteria on motility agar plates (YA + 0.4% agar). The edge of a swarm was picked and inoculated into 100 ml of YA broth and grown from 8 to 16 hr at 37 C. This culture, containing more than 95% motile cells, was used as inoculum.

Routinely, Fernback flasks containing 1 liter of YA broth were used. Cells were grown at 37 C on a rotary shaker and were harvested at late log phase.

Preparation of flagella. Flagella were isolated and purified by ion-exchange chromatography on diethyl-aminoethyl cellulose columns by the method of Martinez (13). Flagellar suspensions were dialyzed exhaustively against deionized water and stored under liquid nitrogen. Protein concentrations were determined by the method of Lowry et al. (12).

Preparation of flagellin. Flagellin solutions were prepared in several ways for immunological analysis and isotope incorporation studies. Routinely, flagella were dissociated by adjusting flagellar suspensions to pH 2.0 with 1 N HCl (1). The resulting solution was then neutralized with 1 N NaOH or dialyzed against several changes of 0.01 M potassium phosphate buffer, pH 7.0. The flagellin solution was then centrifuged at 103,000 × g for 1 hour at 4 C. Flagellin prepared in this manner does not spontaneously polymerize (6).

Preparation of antiflagellar antiserum. Rabbits were hyperimmunized by using the following schedule: 500 μg of purified SL 760 flagella in Freund's complete adjuvant were injected subcutaneously; at 3-week intervals, the rabbits were injected intravenously with 4 mg of Benadryl followed by 500 μg of flagella. About eight injections were given prior to bleeding. All antisera were absorbed with the nonflagellated mutant, SL 4077, to remove cross-reacting O antibodies.

Assay of antiflagellar serum. Sera were routinely assayed for the presence of antiflagellar antibodies by the double-gel diffusion method of Ouchterlony (17). Gels contained 1 g of Noble agar (Difco) and 0.2 g of NaN₃ per 100 ml of phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M potassium phosphate, pH 7.2). This method was also used to detect flagella and flagellin in cell extracts. Flagella and flagellin from strains SL 870 and SL 871 cross-react with antiserum raised against SL 870 flagella.

Three-component assay. The method of Askonas and Rhodes (7) was used to detect ¹⁴C-labeled flagellin in cell extracts. Extracts were dialyzed against PBS and then treated with antiflagellar serum; they were incubated for 1 hr at 37 C and then at 4 C overnight. The flagellin-antibody complexes were precipitated with goat anti-rabbit gamma globulin. Incubations were performed as above. The antigen-antibody pellets were washed three times with PBS, suspended in 5% trichloroacetic acid, collected on Whatman GF/C glass-fiber filters, and counted in a scintillation spectrometer.

Immune hemolysis assay. Extracts were assayed for flagellar antigen by the immune hemolysis method essentially as described by Arquilla and Finn (5).

Sheep red-blood cells were sensitized with flagellin by using bis-diazobenzidine as conjugating agent. All extracts were tested for their ability to bind antibody previously titrated in the hemolytic assay. Flagellar antigen in extracts was demonstrated by its inhibition of hemolysis after the addition of complement.

Preparation of cell-free extracts. The following operations were carried out at 4 C. Cells of each strain were harvested in the early log phase of growth by centrifugation and suspended in buffer. SL 870 and SL 871 suspensions were deflagellated in a Sorvall Omnimixer at top speed for 1 min. The cells were washed with buf-

fer three times and stored under liquid nitrogen. Unless otherwise stated, the cells were broken by hand grinding with alumina. A buffer consisting of 0.01 M tris (hydroxymethyl) aminomethane (Tris; pH 7.8), 0.006 M mercaptoethanol, 0.014 M magnesium-acetate, and 0.06 M KCl (16) was most commonly used and will be referred to as standard buffer. After removal of the alumina, the extracts were centrifuged at 45,000 rev/min (135,000 × g) for 2 hr. This procedure fractionated the extracts into a clear yellow supernatant (45S), a cloudy sediment which was readily suspended in buffer (45LP), and a clear ribosomal sediment (45P).

Isolation and detection of NML: isolation from cells. Bacterial cell paste (25 g, wet weight) was suspended in 100 ml of cold 5% trichloroacetic acid and incubated overnight at 4 C. The precipitate was collected by centrifugation, washed with 5% acid suspended in 5% acid, and heated at 90 C for 30 min to remove nucleic acids. Lipids were removed by extraction with a 1:1 solution of absolute ethanol-diethyl ether at 37 C under reflux. The final sediment was washed three times with ether, dried in air, and hydrolyzed in vacuo at 110 C in 6 N HCl for 20 to 24 hr (18).

Isolation from reaction mixtures. Reaction mixtures were made 5% in trichloroacetic acid, and the precipitate was treated as above.

Isolation from flagella. Purified flagella (1 to 5 mg of protein) were hydrolyzed in vacuo at 110 C in 6 N HCl for 20 to 24 hr. HCl was removed in a flash evaporator.

Detection of NML in hydrolysates. (i) For amino acid analysis, 1 to 5 mg of protein hydrolysates in citrate buffer (pH 2.2; 0.2 N Na⁺) was chromatographed on the 22-cm column of a Beckman 120B amino acid analyzer. The basic amino acids were eluted from the AA-27 resin with 0.35 N sodium citrate buffer (pH 5.28) at 50 C. Flow rates were 75 ml/hr. The ninhydrin color constant for NML was determined by using authentic NML (Calbiochem). (ii) For qualitative identification of NML and isolation of radioactive NML by paper chromatography, protein hydrolysates dissolved in deionized water were chromatographed on ethylene-diaminetetraacetic acid-buffered Whatman 3MM paper by using sodium borate (pH 9.3)-buffered phenol-m-cresol (1:1) as solvent system, by the method of Stewart (19). The acetone dip was omitted, and Whatman 1MM paper was used for tank liners. The papers were developed for 18 hr. The papers were dipped in a solution containing 100 mg of cadmium-acetate, 10 ml of water, 5 ml of glacial acetic acid, 10 ml of acetone, and 1 g of ninhydrin. When hydrolysates containing radioactive material were chromatographed, the amino acids were located with ninhydrin stain, and the chromatogram was cut into strips (1 cm by 1 inch); the strips were placed in scintillation vials and counted in a scintillation spectrometer. In some instances the chromatograms were cut into a 1-inch-wide strip and scanned in a Nuclear-Chicago Actigraph III Counter.

Assay of cell-free extracts. The incorporation of ¹⁴C from ¹⁴C-labeled SAM into either hot 5% trichloroacetic acid-insoluble material or hot 5% trichloroacetic acid-insoluble protein was measured. Reactions were terminated by the addition of an equal volume of cold 10% trichloroacetic acid; the mixtures were heated at 90 C for 30 min; the precipitates were collected onto

Whatman GF/C glass-fiber filter pads and washed with 20 ml of 5% trichloroacetic acid. The filter pads were dried in an oven (110 C for 15 min) and then counted in a scintillation spectrometer. The efficiency of counting (83% for ^{14}C in a Beckman instrument, or 76% in a Nuclear-Chicago spectrometer) was used to correct counts incorporated into picomoles of MNL. The scintillation mixture consisted of 1 g of 2.5 diphenyloxazole + 12.5 mg of 2-p-phenylenebis (5-phenyloxazole) per liter of toluene. In samples where lipids were extracted, the hot 5% trichloroacetic acid-insoluble material was treated with either 1:1 ethanol-ether, as previously described, or with a $CHCl_3$-CH_3OH-H_2O (2:1:0.8) system by the method of Ames (3). The residual protein precipitate was collected and counted as described above.

Acrylamide gel electrophoresis. Flagellin and cell extracts were electrophoresed on 7.0% acrylamide gels by the method of Neville (15), with the exceptions that the upper gel was made 0.22% in acrylamide and the upper buffer was made 0.01% in methyl green (C. Parrish, Ph.D. Thesis, Australian National University, Canberra, 1969). Gels were stained with 0.5% (w/v) Buffalo Black in 7% acetic acid for 3 hr and destained by washing in 10% acetic acid.

For locating radioactive bands, the gels were sliced into 2-mm fractions with a razor blade. The slice was placed on a disc of Whatman 3MM filter paper and homogenized by pressing the filter pad against a piece of parafilm. The homogenized slice was dried in an oven at 110 C before measuring radioactivity.

Radioisotopes. ^{14}CH$_3$-L-methionine (13.6 mCi/mmole) and ^{14}CH$_3$-S-adenosylmethionine (42 mCi/mmole) were purchased from New England Nuclear Corp.

TABLE 1. *Distribution of radioactive epsilon-N-methyllysine (NML) derived from ^{14}CH$_3$-methionine in cell fractions of Salmonella typhimurium*

Strain	Amt of NML[a]				
	Cold 5% TCA[b] soluble (small molecules)	Hot 5% TCA-soluble (nucleic acids)	Diethyl ether-ethanol soluble (lipids)	Insoluble (protein)	flagella
SL 870 (nml⁺fla⁺)	ND[c]	ND	ND	13.4	32.5
SL 871 (nml⁻fla⁺)	ND	ND	ND	10.7	ND
SL 4077 (nml⁺fla⁻)	ND	ND	ND	12.1	

[a] Picomoles of NML derived per milligram of protein.

[b] Trichloroacetic acid.

[c] Not detected. (The limit of detection in this assay is 0.02 pmole of NML/mg of protein.)

RESULTS

Synthesis of NML from methionine by whole cells. To verify that NML in *S. typhimurium* derives its methyl group from methionine, isotope incorporation was followed into various cell fractions of the three strains grown in Spizizen's minimal medium with added histidine and 5 × 10⁻⁶ M ^{14}CH$_3$-methionine (Table 1). A chromatogram of the SL 870 flagella hydrolysate is presented in Fig. 1. The results show that the ^{14}C-labeled NML was detectable in the flagella of strain SL 870 but not of strain SL 871. Further, all three strains contained approximately the same amount of NML per milligram of cellular protein.
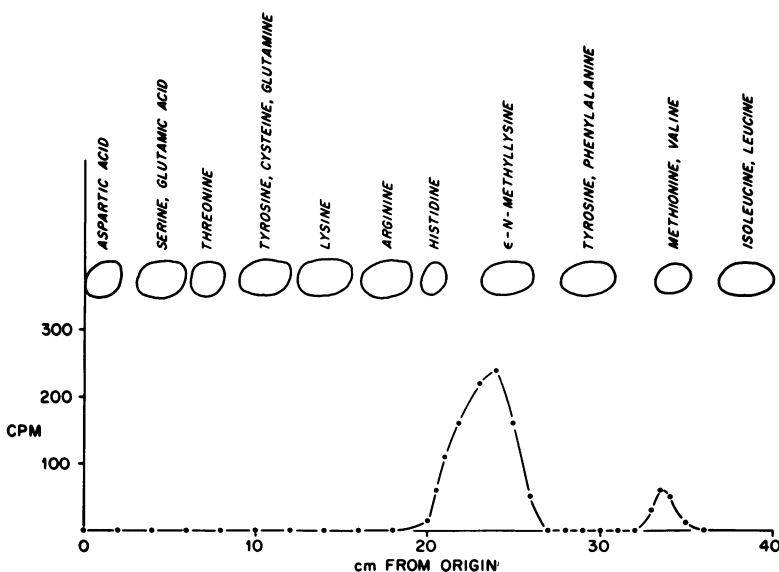

FIG. 1. *Paper chromatography of acid-hydrolyzed S. typhimurium SL 870 flagella labeled with ^{14}CH$_3$-methionine. Acid-hydrolyzed SL 870 flagella was chromatographed on Whatman 3MM paper. The chromatogram was cut into a 1-inch-wide strip and scanned for radioactivity in a Nuclear-Chicago actigraph III strip counter.●, Positions of ninhydrin-positive areas on the chromatogram. The amino acid(s) corresponding to each spot are listed.*

NML synthesis by crude extracts. The ability of the supernatant and sedimented fractions to synthesize NML from SAM was tested (Table 2). The wild-type (SL 870) 45S fraction was approximately twice as active as that from strain SL 871 and almost three times more active than the SL 4077 45S fraction. However, the activities obtained with the 45P fractions of each strain were approximately the same. The LP fractions were without activity. Thus it appears that the bulk of the methylating activity resides in the 45S fraction regardless of strain used.

These results showed striking differences from those obtained when late log-phase cells were used to prepare the cell fractions. Enzyme activity decreases markedly as the cells age. The soluble fraction prepared from late log-phase SL 870 cells were inactive in methylation. Further, the 45P fraction of such cells had only one-half the methylating activity of early log-phase cells. It appears, therefore, that the enzyme activity in early log-phase cells is soluble and that this activity disappears as the cells age.

Effect of chloramphenicol on methylation. The effect of chloramphenicol on the synthesis of NML by a 45S fraction prepared from SL 870 cells was examined. Extract protein (6.8 mg) was incubated with 1.25 μCi of SAM in the presence and absence of 100 μg of chloramphenicol per ml. This concentration of the drug is in 10-fold excess of the concentration required to completely inhibit the growth of the cells. The amount of NML synthesized in the presence and absence of chloramphenicol was the same (140 pmoles of NML/mg of protein). Therefore, protein methylation by these extracts does not require protein synthesis.

Effect of unmethylated substrates on the incor-

TABLE 2. *Methylation of protein by extracts prepared from Salmonella typhimurium*

Strain	Amt of NML[a]	Assay conditions[b]
SL 870		
45S[c]	190	Protein, 10 mg/ml; SAM[d]; 1.25 μCi/ml
45P	12	Protein, 10 mg/ml; SAM; 1.25 μCi/ml
SL 871		
45S	83	Protein, 9.5 mg/ml; SAM, 1.25 μCi/ml
45P	12	Protein, 15.6 mg/ml; SAM, 2.5 μCi/ml
SL 4077		
45S	68	Protein, 8.5 mg/ml; SAM, 2.5 μCi/ml
45P	23	Protein, 21.1 mg/ml; SAM, 2.5 μCi/ml

[a] Picomoles of epsilon-*N*-methyl-lysine (NML) per milligram of protein.

[b] All reaction mixtures were made 0.01 M in potassium phosphate buffer (pH 6.67) and incubated at 37 C for 30 min. Radioactive NML was isolated and counted.

[c] Fraction 45S = a 20,000 X *g* supernatant centrifuged at 133,000 × *g* for 2 hr. Fraction 45P = pellet fraction from extract centrifuged above.

[d] ^{14}CH$_3$-*S*-adenosylmethionine.

poration of SAM by extracts. In these experiments SL 871 flagellin and flagella were used as substrate for methylation. SL 871 is isogenic with SL 870 with the exception of the defective NML gene; therefore, the amino acid sequence of SL 871 flagellin should be identical to that of SL 870 except for its unmethylated lysine residues. Thus, the SL 871 flagellin should serve as substrate for the methylating enzyme obtained from SL 870 or SL 4077. Flagellin or flagella from SL 871 did not stimulate the incorporation of SAM by SL 870 or 4077 extracts nor were the kinetics of SAM incorporation by an SL 4077 extract affected by SL 871 flagellin. The flagellin obtained from *Bacillus subtilis* (totally unmethylated) also had no effect on the kinetics of incorporation of SAM into NML. Bovine serum albumin had no effect. Poly-L-lysine (Sigma Chemical Co.; molecular weight, approximately 200,000) was also tested. However, at concentrations of less than 1 μg/ml, poly-L-lysine precipitated material from the extracts, even after the extracts had been treated with streptomycin to remove nucleic acids.

Characterization of the methylated product. Even though NML$^-$ flagella or flagellin did not stimulate the incorporation of SAM into protein, attempts were made to characterize the radioactive products synthesized by the SL 870 45S fractions.

Reaction mixtures were chromatographed on a Sephadex G-100 column (23 by 2.6 cm). After incubation, the entire reaction mixture was loaded onto the column and eluted with 0.01 M Tris buffer, pH 7.2. The effluent fractions were monitored for protein and radioactivity. A typical elution profile is shown in Fig. 2.

A broad peak of protein was routinely observed. Three radioactive peaks were obtained. The first peak contained 8.2% of the total counts, whereas the second peak contained 34.7% of the total counts. The balance of the input counts were accounted for by a third radioactive peak characterized as SAM. Portions of the first two radioactive peaks were precipitated with an equal volume of 10% trichloroacetic acid, nucleic acid and lipids were extracted, the residual protein was hydrolyzed with 6 N HCl, and the hydrolysates were chromatographed on paper. The extraction procedure removed 95 to 98% of the counts in the peak eluting at the void volume (peak I), and 40 to 50% of the counts from the second peak (peak II). Peak II material after extraction was found to contain approximately 990 pmoles of NML. NML was not detected in peak I.

When the same experiment was carried out by using extracts prepared from SL 871 and SL 4077, similar results were obtained. However,
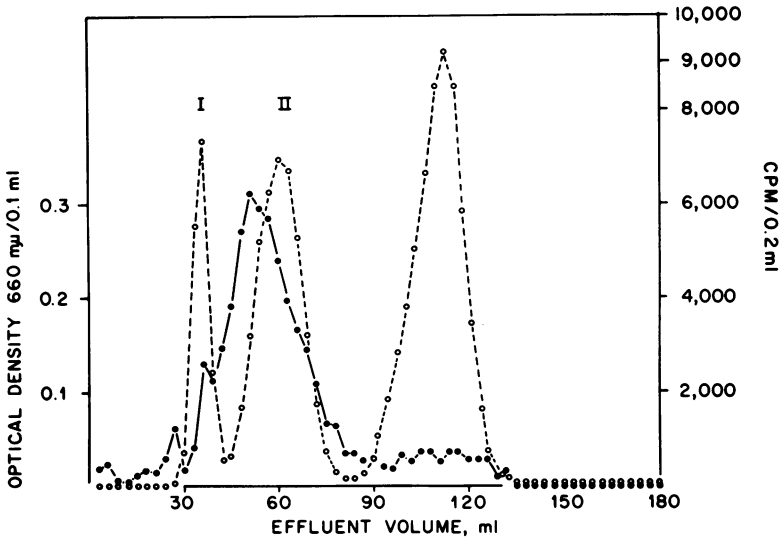
FIG. 2. *Elution profile of a S. typhimurium SL 870 45S fraction from Sephadex G-100. The extract, 20 mg of protein, was incubated with 2.5 µCi of SAM for 30 min at 37 C. The entire reaction mixture was loaded onto a column of Sephadex G-100 (23 by 2.6 cm) and eluted with 0.01 M Tris buffer, pH 7.2. Fractions (3 ml) were collected at a flow rate of 10 ml/hr. Protein was monitored by the method of Lowry et al. (14); 0.2-ml fractions were used to determine radioactivity. The void volume, determined with Blue Dextran, was 35 ml. Symbols: ●, Lowry color, 660 nm per 0.1 ml; ○, counts per minute per 0.2 ml.*

the counts in peak II relative to peak I were greatly reduced. Peak II produced by the mutant extracts contained only 3.6% of the counts, representing a 10-fold reduction compared to SL 870 peak II. As with the SL 870 experiments, NML was detected only in peak II. The experiments are summarized in Table 3.

Since peak I elutes at the void volume, the molecular weight of the radioactive material must be greater than or equal to 100,000. Peak II elutes at a position corresponding to a molecular weight of approximately 50,000. This is in the range of the molecular weight of flagellin (40,000).

Portions of peak I and peak II were analyzed immunologically with antiflagellar serum by the Ouchterlony technique. Flagella and flagellin from strains SL 870 and SL 871 form coincident precipitin lines with antiserum raised against fla-

gella from strain SL 870. Drawings of the gel diffusion plates showing analysis of the Sephadex fractions of SL 870 45S are given in Fig. 3. Both peaks contain O antigen; however, a precipitin band corresponding to flagellar antigen was observed with peak II only. Analysis of SL 4077 peak II, previously shown to contain NML, did not reveal the presence of flagellar antigen. To verify the absence of flagellar antigen in SL 4077, a 45S extract of this strain was analyzed by the more sensitive immune hemolysis technique. The Ouchterlony technique affords detection of 50 µg of flagella or flagellin. The immune hemolysis assay can detect 0.1 to 0.01 µg of flagellin. Again, flagellar antigen was not detected in the 45S fraction from SL 4077 (Fig. 4). Sephadex peaks I and II were also analyzed by the immune hemolysis assay. The same results were obtained as with the Ouchterlony technique. Since peak

TABLE 3. *Analysis of Sephadex G-100 peaks I and II*

Determination	S. typhimurium strain					
	SL 870		SL 871		SL 4077	
	Peak I	Peak II	Peak I	Peak II	Peak I	Peak II
Counts per min	100,416	598,746	75,390	84,928	553,620	95,080
Per cent of total counts per min	8.2 ± 1.5	34.7 ± 3.6	3.2	3.6	18.8	3.6
Per cent of initial counts per min in peak after extraction	3–5	45–55			0.1	40
NML (pmoles)	ND[a]	990		+[b]	ND	220

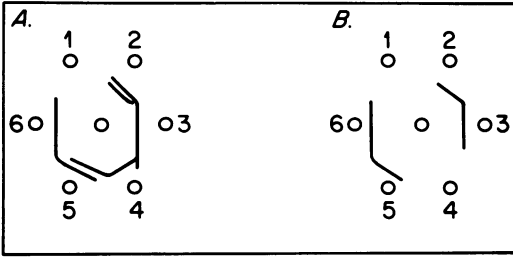[a] Not detectable.

[b] +, NML present but not quantitated.

FIG. 3. *Immunodiffusion analysis of S. typhimurium SL 870 G-100 peaks I and II. (A) Center well contains SL 870 flagellar antiserum. (B) Center well contains SL 870 flagellar antiserum adsorbed with SL 4077 cells to remove anti-O activity. Peripheral wells (A and B): (1) low-molecular-weight radioactive peak (S-adenosyl methionine) obtained by passing a SAM-labeled SL 870 45S extract through a Sephadex G-25 column, 5 μCi; (2) radioactive material eluting at the void volume of the G-25 column, 5 μliters (7,000 counts/min); (3) SL 870 flagella, 50 μg; (4) G-100 peak I, 15,000 counts/min; (5) G-100 peak II, 7,500 counts/min; (6) SL 870 flagella, 50 μg. SL 870 peaks I and II were prepared by passing a reaction mixture of the following composition through a Sephadex G-100 column: 11.6 mg of protein, 5.0 μliters of SAM, 0.01 M phosphate buffer (pH 6.67), in a final volume of 1.0 ml.*

II contains all of the NML and all of the flagellar antigen in the SL 870 extract, these results suggest that flagellin is methylated. Since the experiments give only indirect evidence for this, attempts were made to isolate radioactively labeled flagellin from extracts.

Further characterization of the methylated product: precipitation with antibody. Although flagellin forms a precipitin band with antiflagellar serum, it is not readily precipitated by flagellar antibody in a precipitin test. Since flagellin binds antibody in solution, as demonstrated by the immune hemolysis assay, labeled flagellin, if present in extracts, should bind antiserum to flagella. The flagellin-antibody complexes could then be precipitated with anti-rabbit gamma globulin.

A 45S fraction of SL 870 (6.8 mg) was labeled by incubation at 37 C for 30 min with 1.25 μCi of SAM. The reaction mixture was dialyzed at 4 C until no further radioactivity was found outside the dialysis bag. The extract was then reacted serially with antiflagellar and anti-gamma globulin antisera. The results are given in Fig. 5. Although the numbers of counts precipitated by the antisera increased linearly with increased addition of extract, they represent only about 0.4% of the counts added. The addition of cold-carrier flagellin had little influence on the amount of counts precipitated. Attempts to precipitate labeled flagellin from peak II by this method were not successful.
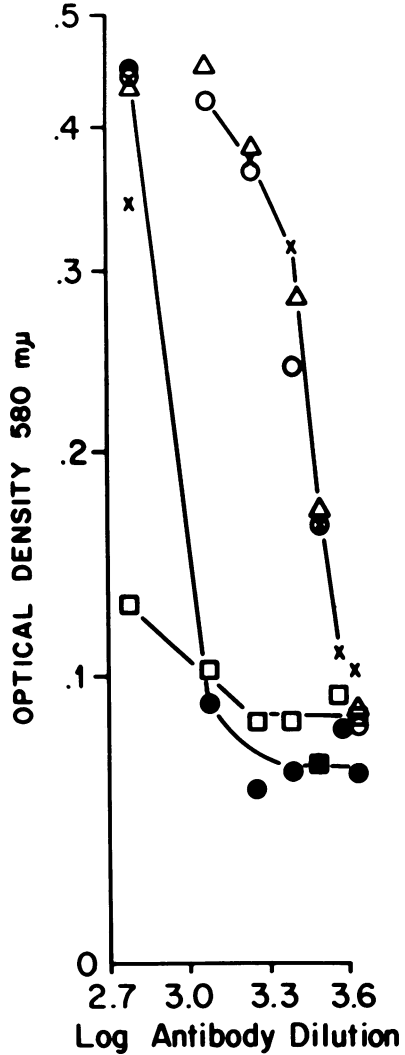

FIG. 4. *Immune hemolysis assay of S. typhimurium SL 4077 45S, SL 870 G-100 peak I, and SL 870 G-100 peak II. Inhibitors were added to the indicated dilutions of antiserum (adsorbed with SL 4077 cells). After incubation at 37 C for 1 hr, and in the cold for 2 hr, red blood cells sensitized with flagellin were added to the tubes. The residual unbound antibody was detected in the presence of excess complement by measuring the extent of hemolysis at 580 nm. Symbols: ×, no inhibitor added; △, SL 4077 45S, 65 μg; ○, SL 870 G-100 peak I, 15,000 counts/min; ●, SL 870 G-100, peak II, 7,500 counts/min; □, SL 870 flagellin, 1 μg.*

Acrylamide gel electrophoresis. The high resolving power of gel electrophoresis was utilized in a further attempt to show that flagellin was actually being methylated. SL 870 extracts were labeled with SAM and then extracted with $CHCl_3$-CH_3OH (2:1). The residual protein was
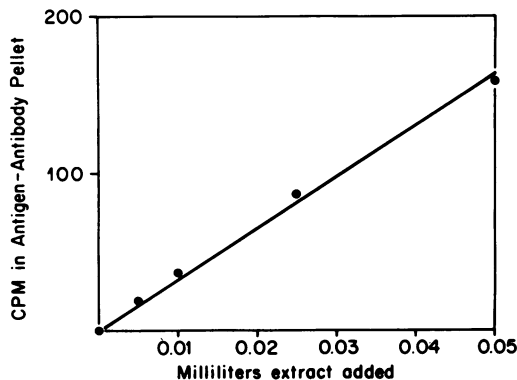
FIG. 5. Precipitation of ¹⁴C-NML with anti-SL 870 flagellar antiserum. Increasing amounts of a SAM-labeled SL 870 45S extract (4,212 counts per min per 0.005 ml) were added to a constant amount of undiluted SL 870 flagellar antiserum. After incubation at 37 C for 60 min and then overnight at 4 C, an equal volume of undiluted-goat-antirabbit gamma globulin was added, and the mixtures were incubated as before. The antigen-antibody pellets were washed three times with PBS. The pellets were suspended, collected on glass filters, and counted.

dissolved in 8 M urea, and this solution was electrophoresed. SL 870 peak II was also run. Figure 6a shows the pattern of radioactivity in the gel for the 45S extract, and Fig. 6b is the pattern for peak II. In the 45S electropherogram a radioactive peak coelectrophoresing with flagellin was found in the gel; however, counts are present in the upper portions of the gel and in bands which had migrated further down the gel. These were not coincident with any one particular protein band. The pattern for G-100 peak II is much the same except that the "flagellin peak" was much reduced in relation to the other peaks. This small peak—or shoulder—was very reproducible.

The acrylamide gel patterns of SAM-labeled SL 870 and SL 4077 crude extracts were also compared. Figure 7 shows the pattern of radioactivity in the gels. The SL 4077 extract lacks the radioactive peak corresponding to flagellin (gel fraction three), but is similar to the SL 870 extract in that the other two major peaks are present.

DISCUSSION

The appearance of NML residues in the flagella of *S. typhimurium* is most likely due to methylation of flagellin prior to its polymerization. This conclusion is supported by the following evidence. (i) Protein was methylated by cell-free extracts that had been centrifuged at speeds sufficient to remove flagella. (ii) The NML syn-
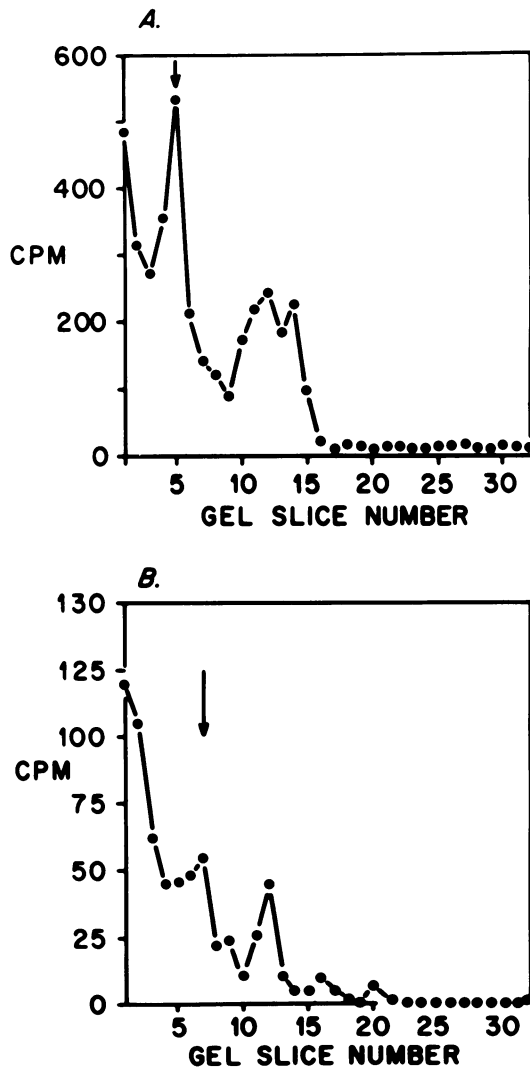

FIG. 6. Profile of radioactivity in acrylamide gels of S. typhimurium SL 870 45S and G-100 peak II. (A) An SL 870 45S extract, 11.7 µg of protein, was labeled with 5.0 µCi of SAM for 30 min at 37 C. The extract was then extracted with CHCl₃-CH₃OH (2:1) until the radioactivity in the CHCl₃ phase reached a constant low level. The residual protein was dissolved in 8 M urea and run for 4 hr at 2.5 ma/tube. Fifty microliters (31,000 counts/min) was placed on the gel. The arrow indicates the position of the flagellin band determined independently. (B) A 1.0-ml portion from a reaction mixture containing 10 µCi of SAM, 43 mg of an SL 870 45S extract, in a total volume of 3.5 ml (incubated at 37 C, 30 min) was passed through a Sephadex G-100 column. Peak II was isolated and concentrated by lyophilization. Fifty microliters (1,000 counts/min) was placed on the gel; electrophoresis was for 3.75 hr and at 2.5 ma/tube. The arrow indicates the position of the flagellin band.
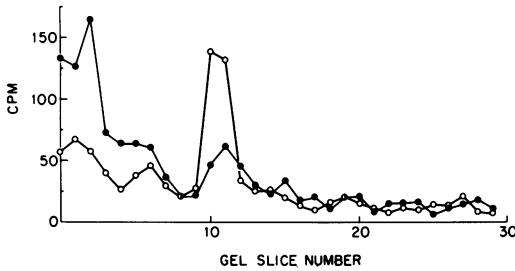
FIG. 7. *Profile of radioactivity in acrylamide gels of SL 870 and SL 4077 crude extracts. Crude extract protein (10 mg) was incubated with 3.75 μCi of SAM for 30 min at 37 C in phosphate buffer (pH 6.85; 0.01 M). Lipids were removed with* $CHCl_3$-CH_3OH; *the residual protein was dissolved in 8 M urea and electrophoresed. Symbols:* ●, *SL 4077;* ○, *SL 870.*

thesized from SAM by cell-free extracts is found only in protein of molecular weight 40,000 to 60,000. (iii) Flagellar antigen is found only in this protein fraction. (iv) NML is not found in the hot 5% trichloroacetic acid-soluble fraction or the cold 5% trichloroacetic acid-soluble fraction of extracts labeled with SAM or cells grown in the presence of growth-inhibiting concentrations of chloramphenicol. (v) The amount of NML synthesized in the presence of chloramphenicol is equal to that made in its absence. (vi) Radioactivity can be precipitated out of SAM-labeled extracts with antibody against flagellin. (vii) Acrylamide gel electrophoresis of labeled extracts and peak II from a Sephadex G-100 chromatogram resolves a peak of radioactivity that coelectrophoreses with flagellin. This peak is not present in SAM-labeled extracts of the *nml⁺fla⁻* mutant.

Comparison of NML synthesis by the two mutant strains (SL 871 and SL 4077) with the wild type (SL 870) suggests that one or more proteins, in addition to flagellin, are methylated. The results supporting this conclusion are as follows. (i) Deflagellated cells of each strain grown on ¹⁴CH₃-methionine contained approximately the same amount of NML per milligram of protein. Radioactive NML was not found in the flagella of SL 871. (ii) Cell-free extracts prepared from each strain synthesized NML from SAM. (iii) Synthesis of NML by 45P fractions prepared from cells of all three strains was approximately the same. However, the wild-type 45S fraction was more active than the mutant 45S fractions. (iv) More than one radioactive peak was obtained when extracts of SL 870, labeled with SAM, were electrophoresed on acrylamide gels. (v) Only one-tenth of the radioactive NML in SL 870 extracts could be precipitated with antibody to flagella in the three-component assay.

Although SL 871 (*nml⁻fla⁺*) methylates pro-

tein, as mentioned above, NML is never found in its flagella. One cannot ascribe the lack of methylation of flagellar protein by SL 871 to an altered structure of flagellin since fingerprints of tryptic digests of SL 870 and SL 871 flagellin are indistinguishable (N. Lundh and R. J. Martinez, *unpublished data*). Further, strain SL 4077 (*nml⁺ fla⁻*) does not contain detectable flagellar antigen. Yet SL 4077 forms NML from SAM (see Table 1). These data suggest, therefore, that there must be one gene for methylation of flagellin and one or more genes for methylating other proteins. The genetic analysis of Stocker and coworkers (20) considered the presence or absence of NML in flagella but not in other cellular proteins. Thus, they did not eliminate the possible existence of other methylating genes.

The difference in specific activity of methylating ability between SL 870 and the two mutants may be explained by the following argument. SL 870 possesses both the substrates and enzymes for modifying flagellin and other cellular protein(s). SL 871, however, is devoid of the NML gene for flagellin, whereas SL 4077 is devoid of the substrate for the flagellin-specific methylating enzyme.

SL 871 flagellin should be identical in primary sequence to SL 870 flagellin except for the absence of methylated lysine residues. Thus, it would be expected that SL 871 flagellin should serve as a substrate for the flagellin-methylating enzyme of strain SL 4077 (*nml⁺ fla⁻*). However, the addition of the NML⁻ flagellin prepared by several methods to extracts of SL 4077 did not stimulate the incorporation of SAM. Attempts were also made to separate the flagellin-methylating enzyme from the endogenous "flagellin" substrate so that an enhancement of methylation might be observed upon addition of exogenous flagellin. All attempts were unsuccessful.

In summary, the data presented support the contention that, in addition to flagellin, other cellular protein(s) are methylated in *S. typhimurium*. Further, the data also support the view that these alkylations are mediated by more than one enzyme—the product of the *nml* gene is specific for flagellin and one, or more than one, other methylating enzyme for the modification of one, or more than one, other cellular proteins.

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