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Methylation of the Ribosomal Proteins in *Escherichia coli.* Nature and Stoichiometry of the Methylated Amino Acids in 50S Ribosomal Proteins†

C. N. Chang and F. N. Chang*

ABSTRACT: Methylated ribosomal proteins from *Escherichia coli* 50S subunit are localized by growing cells in a medium containing [1-^{14}C]methionine and [^{3}H-*methyl*]-methionine and comparing the ^{3}H/^{14}C ratio for each of the 50S ribosomal proteins. The following proteins are methylated: L11, L1, L3, L5,L7, L8, L9, L12, L18, and L33. The nature and stoichiometry of the methylated amino acid(s) in each of the methylated proteins are determined. Protein L11 is the most heavily methylated of all the 50S subunit proteins. This protein has previously been implicated in the peptidyl transferase reaction during protein synthesis (K. H. Nierhaus and V. Montejo (1973), *Proc. Nat. Acad. Sci. U. S. 47,* 1588–1602). Three proteins (L1, L3, and L5) have intermediate levels of methylation and contain about 0.4–0.6 methyl groups each per molecule of protein. Five other proteins (L7, L8, L9, L12, and L18) are also methylated to a slight extent (~0.1 methyl group/molecule of protein). One unknown methylated neutral amino acid was detected in protein L11 and at least one and possibly two other unidentified methylated amino acids appeared to be present in protein L33.

Methylated amino acids have been reported to be present in ribosomal proteins (Comb *et al.*, 1966; Terhorst *et al.*, 1972, 1973; Chang *et al.*, 1974; Alix and Hayes, 1974). Terhorst *et al.* (1972, 1973) showed that 50% of one lysine residue from two closely related ribosomal proteins, L7 and L12, was methylated to ϵ-*N*-monomethyllysine in *Escherichia coli* MRE600. Recently we have detected the presence of approximately 0.8–0.9 molecule of ϵ-*N*-trimethyllysine in protein L11 (Chang *et al.*, 1974). Similar observations have been reported by Alix and Hayes (1974). A rapid method for the localization of the methylated ribosomal proteins in *E. coli* has been described (Chang *et al.*, 1974; Chang and Chang, 1974). Using this method we have shown that methylation of ribosomal proteins occurs predominately in 50S subunit proteins.

Methyl-deficient 50S ribosomal particles have been obtained from an *E. coli rel*$^{-}$ *met*$^{-}$ strain after starvation for methionine. Such particles can be methylated *in vitro* using enzymes derived from the 0.5 M KCl wash of wild type ribosomes (Chang and Chang, 1974). Protein L11 and, to a much lesser extent, proteins L1, L3, and L5 were found to be methylated *in vitro*. The methylated amino acids in protein L11 have been characterized and shown to be predominately ϵ-*N*-trimethyllysine (Chang and Chang, 1974). Since most of the methylated 50S ribosomal proteins (except protein L11 and possibly protein L33) contain only small amounts of methylated amino acids, a sensitive procedure

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has to be developed for their identification. We now report the determination of the nature and stoichiometry of the methylated amino acids in the methylated 50S proteins from cells grown in the presence of [^{14}C-*methyl*]methionine.

Materials and Methods

[1-^{14}C]Methionine (specific activity 54 Ci/mol) and [^{3}H-*methyl*]methionine (specific activity 2.4 Ci/mmol) were obtained from Amersham/Searle. *E. coli* Q13 carrier cells were purchased from General Biochemical Inc. Lysine, arginine, histidine, methionine, ϵ-*N*-monomęthyllysine (MML),[1] 1-methylhistidine (1-MH), 3-methylhistidine (3-MH), methionine sulfoxide (M-SO), and methionine sulfone (M-SO_2) were obtained from Sigma Chemical Co. N^G-monomethylarginine (MMA) and ϵ-*N*-dimethyllysine (DML) were products of Cyclo Chemical Co. ϵ-*N*-Trimethyllysine (TML), N^G,N^G-dimethylarginine (unsym-DMA or uDMA), and N^G,N'^G-dimethylarginine (sym-DMA or sDMA) were purchased from Calbiochem. ϵ-Dnp-lysine was obtained from Schwarz/Mann. Prior to either high-voltage paper electrophoresis or descending paper chromatography, TML, MMA, uDMA, and sDMA were dissolved in water and passed through Dowex 1 columns to remove the conjugated dyes. These amino acids were then concentrated by lyophilization and dissolved in water to give final concentrations of 10 mg/ml.

Escherichia coli Q13 was used throughout the work. For double labeling experiments, cells were grown in 10 ml of a Tris-buffered medium (0.1 M Tris-HCl, 1 mM $MgCl_2$, 0.01 mM $FeCl_3$, 0.1 mM $CaCl_2$, 1 mM KH_2PO_4, and 0.32 mM Na_2SO_4 adjusted to a final pH of 7.4) supplemented with 0.3% glucose, 2 μg/ml of thiamine, and 15 μg/ml of each of 20 amino acids except methionine; 15 μCi of [1-^{14}C]methionine and 150 μCi of [^{3}H-*methyl*]methionine were then added to give a final methionine concentration of 7.5 μg/ml. After harvesting the cells at late log phase, 150 mg of carrier Q13 cells were added and the cells were disrupted with a French press in a buffer containing 0.01 M Tris-HCl (pH 7.8), 0.1 mM $MgCl_2$, and 0.05 M KCl; 50S ribosomal subunits were prepared by layering 2 ml of the extract on top of a 60-ml 5–20% sucrose gradient in a SW 25.2 rotor. The ribosomal subunits were then concentrated by ethanol precipitation (Staehelin *et al.*, 1969). After dissolving the pellet in a buffer containing 0.01 M Tris-HCl (pH 7.8), 0.1 mM $MgCl_2$, and 0.05 M KCl, ribosomal proteins were prepared by the rapid addition of 2 volumes of glacial acetic acid according to the procedure of Hardy *et al.* (1969). The supernatant protein solution was lyophilized to dryness and dissolved in 0.15 ml of the sample gel solution used for the first dimension run of the two-dimensional polyacrylamide gel electrophoresis procedure of Kaltschmidt and Wittmann (1970). After electrophoresis and staining of the gel, regions for the individual proteins were cut out of the gel and counted according to the procedure of Nashimoto *et al.* (1971).

To analyze the methylated amino acids in each protein, the cells were grown in the medium described previously for the double labeling experiment with 50 μCi of [^{14}C-*methyl*]methionine as the sole radioactive source (final concentration 7.5 μg/ml). After the addition of 100 mg of carrier Q13 cells, the cells were disrupted with a French press in a buffer containing 0.01 M Tris-HCl (pH 7.8), 10 mM $MgCl_2$, and 0.05 M KCl; 70S ribosomes were prepared by a modification (Cox *et al.*, 1964) of the procedure of Nirenberg and Matthaei (1961) and the final ribosomal pellet was resuspended in the above buffer; 60 A_{260} units of the 70S ribosomes in 0.5 ml of buffer was then adjusted to 0.1 M $MgCl_2$, and the ribosomal proteins were prepared according to the procedure of Hardy *et al.* (1969). The procedures for the two-dimensional polyacrylamide gel electrophoresis and localization of the proteins from the gels were the same as those described in the above section for the double labeling experiments.

Proteins were cut out of the gels and extracted overnight by gentle shaking with 6 ml of 0.01 M sodium phosphate buffer (pH 6.8) containing 0.1% sodium dodecyl sulfate (SDS). This was followed by dialysis against three changes of water to remove the excess dye and SDS. The dialysates were filtered to remove the gel and then lyophilized to dryness. The proteins were then hydrolyzed with 2 ml of redistilled 6 N HCl in sealed tubes at 110° for 24–48 hr. The hydrolysates were evaporated to dryness under reduced pressure and redissolved in 2 ml of water. This was again followed by evaporation to dryness. The final pellets were dissolved in small amounts of water for the analysis of the methylated amino acids.

The methylated amino acids were separated by high-voltage paper electrophoresis in a buffer containing pyridine–acetic acid–water (25:1:225), pH 6.5 (Offord, 1966). This system has previously been used by Reporter and Corbin (1972) to separate 3-MH, 1-MH, uDMA (which may partially overlap with sDMA under their conditions), and the separation of TML from MML. We have modified their procedure by using a longer paper (110 cm). MMA and DML were also included as standards. Figure 1 shows the schematic drawing of the separation of each of the eight methylated amino acids. It is evident that when the longer paper was used, the complete separation of 3-MH, 1-MH, MMA, uDMA, and sDMA is achieved. The three methylated lysines migrate together. One high-voltage electrophoresis run allows the identification of the nature of the methylated amino acid if the methylation occurs either in histidine or arginine residues.

While analyzing the methylated amino acids in protein L11 by ion-exchange column (pH 5.84), we have observed that in addition to radioactive methionine (and its derivatives), an unidentified methylated neutral amino acid(s) was also present (Chang *et al.*, 1974). The unidentified methylated neutral amino acid(s) appeared to migrate together with methionine (and its derivatives) when high-voltage electrophoresis was carried out at pH 6.5 described above (Chang *et al.*, 1974). A similar methylated neutral amino acid has been reported by Alix and Hayes (1974). In order to detect the possible presence of this methylated neutral amino acid(s) in the 50S proteins we have also carried out high-voltage electrophoresis in sodium borate buffer at pH 9.3. Methionine, methionine sulfoxide, methionine sulfone, and the unidentified methylated amino acid were clearly separated by this procedure (see Figure 7). All basic amino acids migrated together. This unknown methylated neutral amino acid(s) has also been detected by ion-exchange column using 0.2 N sodium citrate buffer (pH 3.25) where it migrated slightly faster than methionine sulfoxide

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; MML, ϵ-*N*-monomethyllysine; DML, ϵ-*N*-dimethyllysine; TML, ϵ-*N*-trimethyllysine; MMA, N^G-monomethylarginine; uDMA, N^G,N^G-dimethylarginine; sDMA, N^G,N'^G-dimethylarginine; 1-MH, 1-methylhistidine; 3-MH, 3-methylhistidine; M-SO, methionine sulfoxide; M-SO_2, methionine sulfone; SDS, sodium dodecyl sulfate.

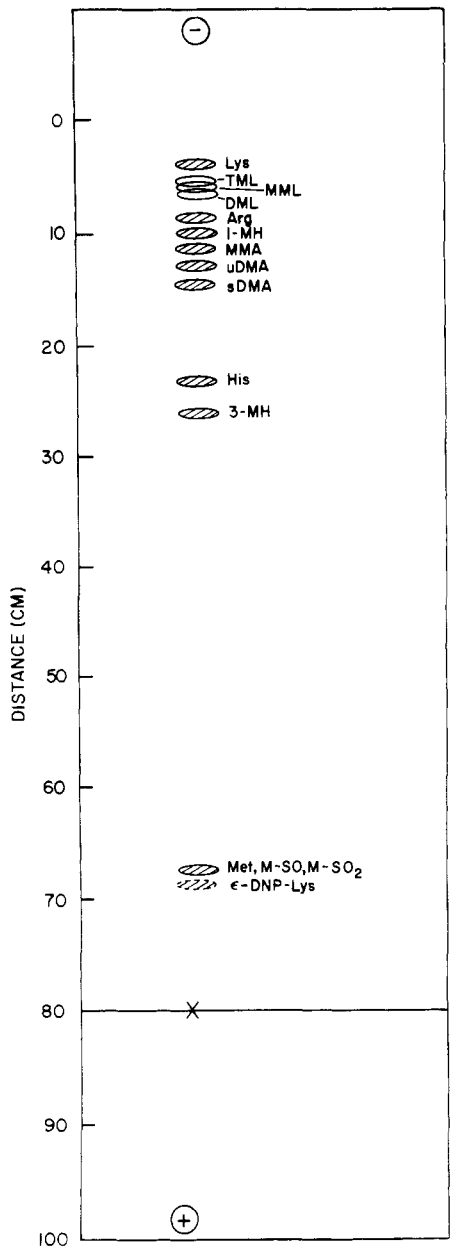

FIGURE 1: Separation of methylated amino acids by high-voltage paper electrophoresis. Approximately 10 μg of each amino acid was applied to a Whatman No. 3MM paper (15 × 110 cm) at 20 cm from one end and was subjected to electrophoresis for 3.5 hr at 4400 V in a buffer containing pyridine–acetic acid–water (25:1:225, v/v), pH 6.5. ϵ-Dnp-lysine was used as a marker for the electrophoresis. After electrophoresis, the paper was sprayed with 0.4% ninhydrin in acetone and heated to 70° for 5 min to locate the amino acids.

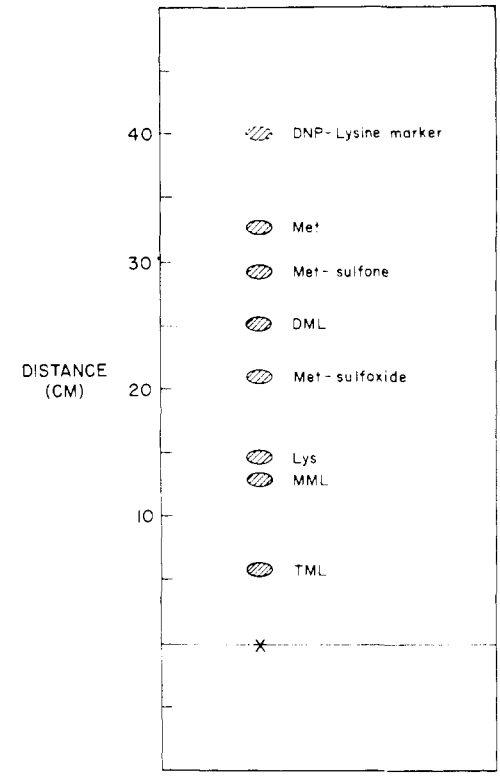

FIGURE 2: Separation of methylated lysines by descending paper chromatography. Approximately 8 μg of each amino acid was applied to a Whatman No. 1 paper. The solvent system was pyridine–acetone–3 M NH_4OH (50:30:25, v/v). Time of chromatography was 13 hr. ϵ-Dnp-lysine was used as a marker. After chromatography, the paper was sprayed with ninhydrin as described in Figure 1.

(F. N. Chang, C. N. Chang, and M. Schwartz, unpublished observations). Its nature is currently under investigation. The two electrophoresis procedures mentioned above (pH 6.5 and pH 9.3) do not separate the three methylated lysines (MML, DML, and TML). However, their separation can be achieved by descending paper chromatography procedures using the following solvent systems: (a) pyridine–acetone–3 M NH_4OH (50:30:25, v/v) (Kakimoto and Akazawa, 1970); (b) phenol–*m*-cresol–sodium borate buffer (pH 9.3) (190:155:45, v/v) (Stewart, 1963). Since solvent system (a) gives much better resolution for these three methylated lysines, we have used this system extensively. We have also used solvent system (b) to verify the presence of ϵ-*N*-trimethyllysine in several proteins. Figure 2 shows the separation of the three methylated lysines using solvent system (a). The other five methylated amino acids (MMA, uDMA, sDMA, 1-MH, and 3-MH) all migrate between lysine and methionine sulfoxide and therefore do not overlap with any of the methylated lysines. The combination of high-voltage paper electrophoresis and descending paper chromatography allows the identification of all eight methylated amino acids.

Results

By growing cells in a medium containing both [1-^{14}C]methionine and [^{3}H-*methyl*]methionine and comparing the $^3H/^{14}C$ ratio for each of the ribosomal proteins obtained from the 70S ribosomes, we have previously shown that methylation of the ribosomal proteins in *E. coli* occurs predominately (if not exclusively) in the 50S subunit proteins (Chang and Chang, 1974; Chang *et al.*, 1974). The methyl esters of glutamic and aspartic acids were not analyzed since they would not survive the high pH of the first dimension run (pH 8.6) of the two-dimensional polyacrylamide gel electrophoresis procedure. The double labeling experiment is very sensitive for proteins with low methionine content (such as ribosomal proteins) and it provides a quick procedure for localizing the proteins that are methylated. Table I shows the $^3H/^{14}C$ ratio for each of the proteins from the 50S ribosomal subunit. It is evident that proteins L1, L3, L5, L7, (L8 + L9), L11, L12, L18, and L33 were methylated. This is in agreement with our previous results using 70S ribosomes (Chang *et al.*, 1974). Due to the fact that proteins L8 and L9 usually migrated together (Kaltschmidt and Wittmann, 1970) and the radioactive counts in methylated amino acids from proteins L7, L12,

L18, and L33 were too low when 50S ribosomal subunits were used, we have analyzed the methylated amino acids present in each of the above proteins from 70S ribosomes obtained from cells grown in the presence of [^{14}C-*methyl*]methionine. Proteins L12 and S6 migrated together when 70S ribosomes were used as starting materials.

In order to identify the presence of methylated derivatives of lysine, arginine, and histidine in the above methylated proteins, high-voltage electrophoresis was initially carried out in pyridine–acetic acid–water system (pH 6.5, see Figure 1). The methylated lysines were then further identified by descending paper chromatography (solvent system (a), see Figure 2). Figures 3 and 4 show such analyses for proteins L1, L3, L5, and L7 by high-voltage paper electrophoresis (pH 6.5) and descending paper chromatography (solvent system (a)), respectively. It is clear that protein L1 contains methylated lysine(s), and a compound with the same mobility as N^G,N'^G-dimethylarginine in our high-voltage paper electrophoresis system (Figure 3). The methylated lysines were identified to be both ϵ-*N*-trimethyllysine and ϵ-*N*-monomethyllysine. Protein L3 contains predominately ϵ-*N*-trimethyllysine. The methylated amino acids in protein L5 are ϵ-*N*-trimethyllysine and a trace amount of ϵ-*N*-monomethyllysine. Protein L7 contains ϵ-*N*-monomethyllysine and a compound with the same mobility as ϵ-*N*-trimethyllysine using the descending paper chromatography system (Figure 4).

Figures 5 and 6 show the analyses of the methylated amino acids by high-voltage paper electrophoresis (pH 6.5) and descending paper chromatography (solvent system (a)), respectively, for proteins L9, L11, (L12 + S6), and L18. Protein L9 contains primarily ϵ-*N*-trimethyllysine. Protein L11 contains predominately ϵ-*N*-trimethyllysine and a small amount of a compound with the same mobility as N^G,N'^G-dimethylarginine. The mixture of proteins L12 and S6 contain a trace amount of ϵ-*N*-monomethyllysine. In other preparations we have detected the presence of trace amounts of both ϵ-*N*-monomethyllysine and ϵ-*N*-trimethyllysine in the mixture of proteins L12 and S6. The methylated amino acid in protein L18 is ϵ-*N*-trimethyllysine. Although the data are not presented here, we have determined the methylated amino acid in protein L8 and found that it is primarily ϵ-*N*-monomethyllysine. In all of the above proteins we did not detect the presence of significant amounts of ϵ-*N*-dimethyllysine, which would migrate between methionine sulfoxide and methionine sulfone in solvent system (a) (Figure 2). However, these results could not rule out the possibility of the presence of trace amounts of ϵ-*N*-dimethyllysine in these proteins.

In order to ascertain whether any of the above proteins contain the unidentified methylated amino acid(s) observed previously in protein L11 (Chang *et al.*, 1974; Alix and Hayes, 1974), high-voltage electrophoresis was also carried out in sodium borate buffer (pH 9.3). This system separates the unidentified methylated neutral amino acid(s) from the methylated basic amino acid and methionine (and its derivatives). Figure 7 shows that protein L11 contains the unidentified methylated amino acid(s) and the ratio of this unidentified methylated neutral amino acid to ϵ-*N*-trimethyllysine is about 0.6:1. Proteins L1, L3, and L5 also contain a significant amount of the unidentified neutral methylated amino acid(s) (Table II). Small amounts of the unidentified methylated amino acid(s) are also present in other proteins (L7, L8, L9, Li2, and L18). Since we have also detected small amounts of the unidentified methylated amino acid(s)

Table I: Localization of Ribosomal Protein from the 50S Subunits which are methylated.

Protein	^{14}C (cpm)	^{3}H (cpm)	^{3}H/^{14}C	Δ^{3}H/^{14}C[a]
L1	1546	5109	3.31	+0.51
L2	400	919	2.30	−0.50
L3	621	2092	3.37	+0.57
L4	577	1598	2.77	−0.03
L5	482	1610	3.34	+0.54
L6	229	661	2.88	+0.08
L7	92	302	3.28	+0.58
L8 + L9	166	772	4.65	+1.85
L10	865	2188	2.53	−0.27
L11	1289	6392	4.96	+2.16
L12	49	161	3.29	+0.59
L13	694	1723	2.83	+0.03
L14	251	653	2.60	−0.20
L15	743	1872	2.52	−0.28
L16	587	1741	2.97	+0.17
L17	754	1894	2.51	−0.29
L18	181	771	4.26	+1.46
L19	245	464	1.90	−0.90
L20	1	18		
L21	223	704	3.15	+0.35
L22	777	2266	2.92	+0.12
L23	303	964	3.18	+0.38
L24	26	72		
L25	554	1604	2.90	+0.10
L26	388	909	2.34	−0.46
L27	30	112		
L28	96	206	2.15	−0.65
L29	292	850	2.91	+0.11
L30	386	847	2.19	−0.61
L31	27	69		
L32	213	412	1.93	−0.87
L33	10	257	25.7?	+22.9?

[a] The ^{3}H/^{14}C ratio for the total unfractionated ribosomal protein was 2.80. Δ^{3}H/^{14}C for any protein is ^{3}H/^{14}C − 2.80. Proteins where methyl groups are present in excess of the methionine present are underlined.

(up to 1–2% of total methionine counts) when [^{14}C-*methyl*]methionine was hydrolyzed at 110° for 40 hr, we have not ruled out the possibility that the methylated neutral amino acid(s) present in the above proteins (with the possible exceptions of L11, L3, and L5) is an artifact. Protein L33, on the other hand, contains a new radioactive peak which is different from any of those present in protein L11 (Figure 7). This new radioactive peak in protein L33 appears to contain two unidentified methylated amino acids when protein L33 hydrolysate was analyzed by ion-exchange column (F. N. Chang, C. N. Chang, and M. Schwartz, unpublished data). The nature of these unidentified methylated amino acids are currently under investigation. Protein L33 may also contain a trace amount of either monomethylarginine or 1-methylhistidine. A summary of the methylated amino acids present in the above proteins is presented in Table II. The presence of ϵ-*N*-trimethyllysine in proteins L1, L3, L5, L9, and L11 was also verified by descending paper chromatography using solvent system (b). We have also previously identified the presence of ϵ-*N*-trimethyllysine in protein L11 by the use of the amino acid analyzer (Chang *et al.*, 1974). Because of low counts we have not been able to identify N^G,N'^G-dimethylarginine in either proteins L1 or L11 using the amino acid analyzer.

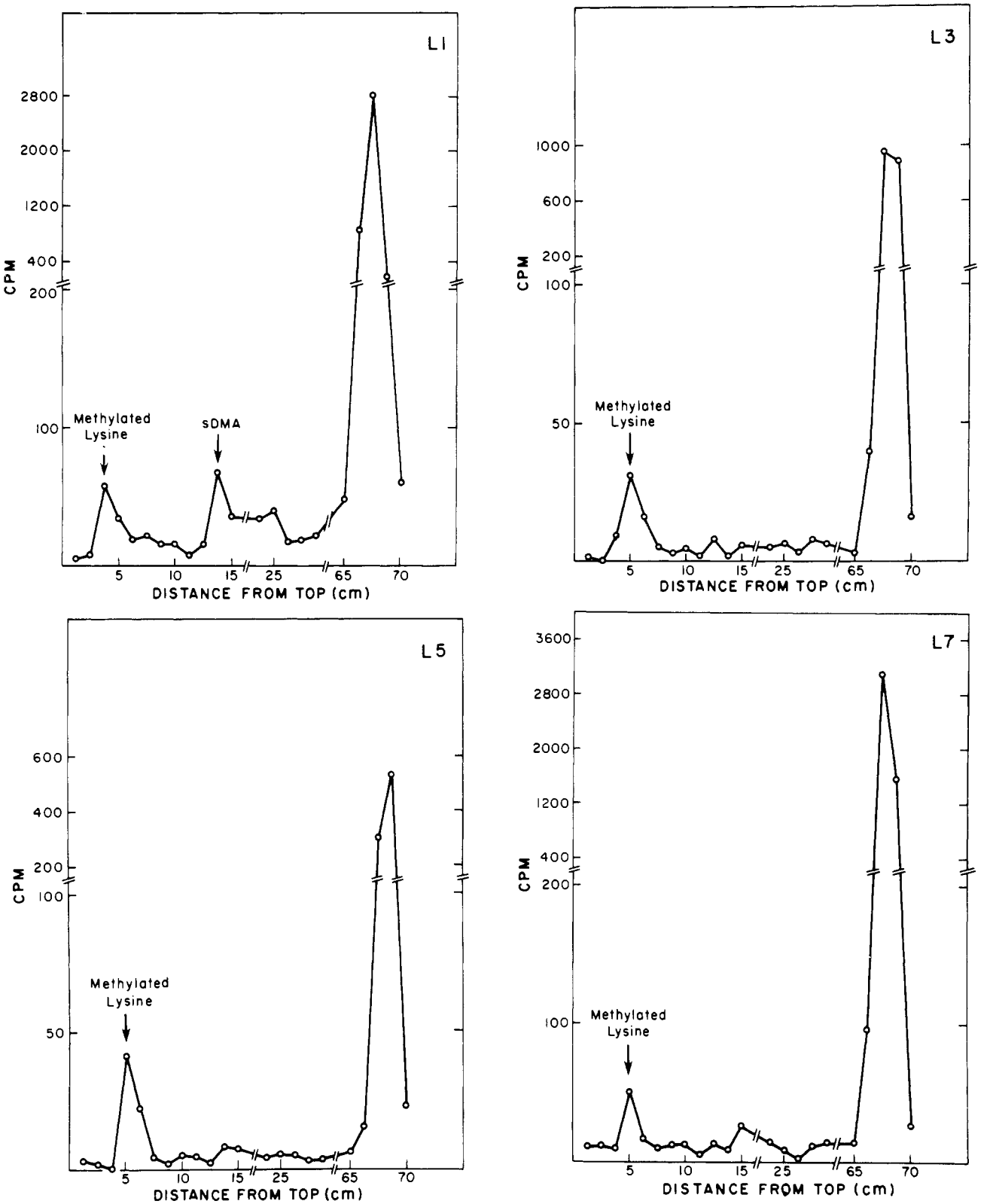

FIGURE 3: Analysis of methylated amino acids derived from proteins L1, L3, L5, and L7 by high-voltage paper electrophoresis; 20 μl of each protein hydrolysate (described under Materials and Methods) was applied to a Whatman No. 3MM paper (40 × 110 cm) at 20 cm from one end together with several of the following amino acids: MML, DML, TML, MMA, sDMA, uDMA, 1-MH, 3-MH, Lys, Arg, His, Met, M-SO, and M-SO_2. The conditions for high-voltage electrophoresis and spraying with ninhydrin were described in the legend of Figure 1. The paper was then cut into 1.25-cm strips and counted in a toluene-based scintillator fluid (4 g of Omniflour/l. of toluene). Occasionally we have observed 1-MH to be migrating slower than sDMA.

Hence its identification can only be considered tentative at present.

Since [^{14}C-*methyl*]methionine was used as a methyl donor, it is possible to determine the stoichiometry of the methylated amino acids present in the proteins from the mole % methionine and the molecular weight of each protein. The stoichiometry of the methylated amino acids in proteins L1, L3, L5, L7, L8, L9, L11, (L12 + S6), and L18

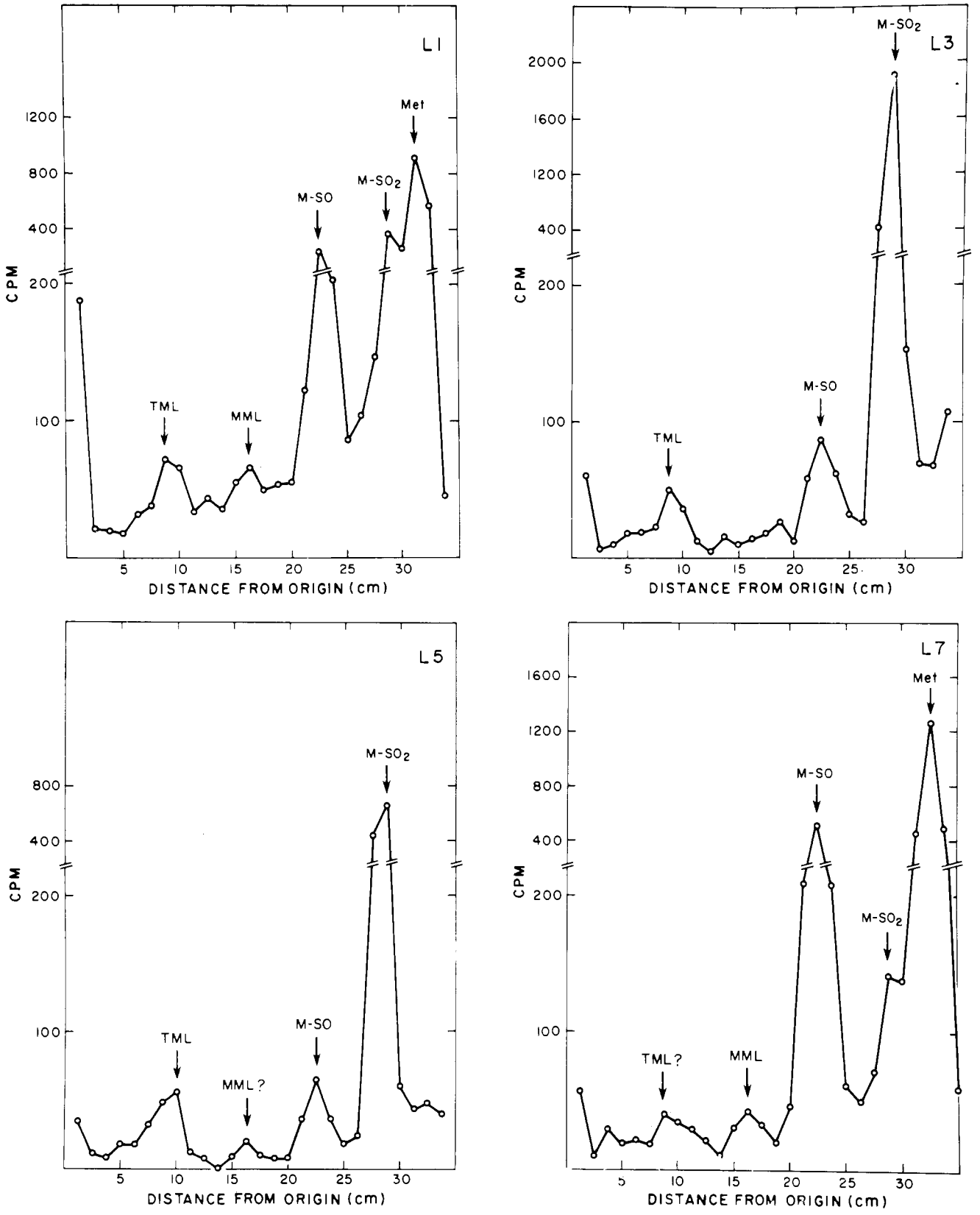

FIGURE 4: Analysis of methylated lysines derived from proteins L1, L3, L5, and L7 by descending paper chromatography using solvent system (a); 15 μl of each protein hydrolysate (described under Materials and Methods) was applied to a Whatman No. 1 paper together with several of the following amino acids: MML, DML, TML, Met, M-SO, and M-SO_2. The conditions for paper chromatography were described in Figure 2. After chromatography and spraying of the paper with ninhydrin, the paper was cut into 1.25-cm strips and counted as described in Figure 3.

is presented in Table II. It is evident that protein L11 contains about five methyl groups per molecule of protein. Protein L5 contains approximately 0.6 methyl group per molecule of protein. Proteins L1 and L3 contain about 0.4 methyl group each per molecule of protein. The other methylated proteins (except L33) contain approximately 0.1 methyl group per molecule of protein. Due to the fact that protein L33 does not contain methionine (Kaltschmidt *et al.*, 1970),

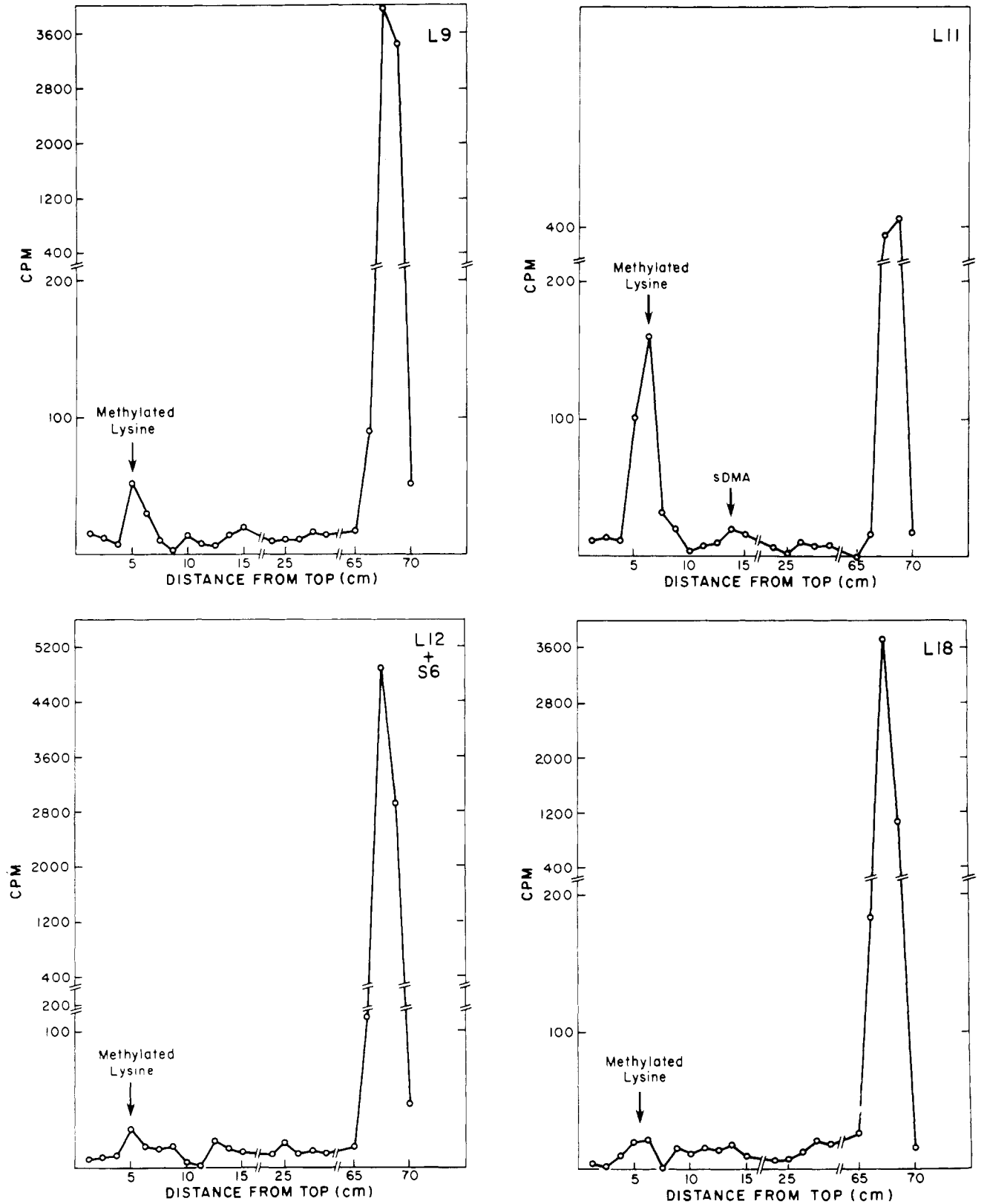

FIGURE 5: Analysis of methylated amino acids derived from proteins L9, L11, L12 + S6, and L18 by high-voltage paper electrophoresis. The conditions were the same as those described in Figure 3 except protein hydrolysates from L9, L11, L12 + S6, and L18 were used.

the stoichiometry of the two unidentified methylated amino acids in protein L33 is unknown at present.

Discussions

Terhorst *et al.* (1972) have shown previously that proteins L7 and L12 from *E. coli* MRE600 contained approximately 0.5 molecule of ϵ-*N*-monomethyllysine/molecule of protein. Our studies have confirmed the presence of ϵ-*N*-monomethyllysine in both proteins except that the magnitude of methylation was much less (~0.05 molecule per molecule of protein). In addition, protein L7 and some preparations of protein L12 derived from *E. coli* Q13 seem

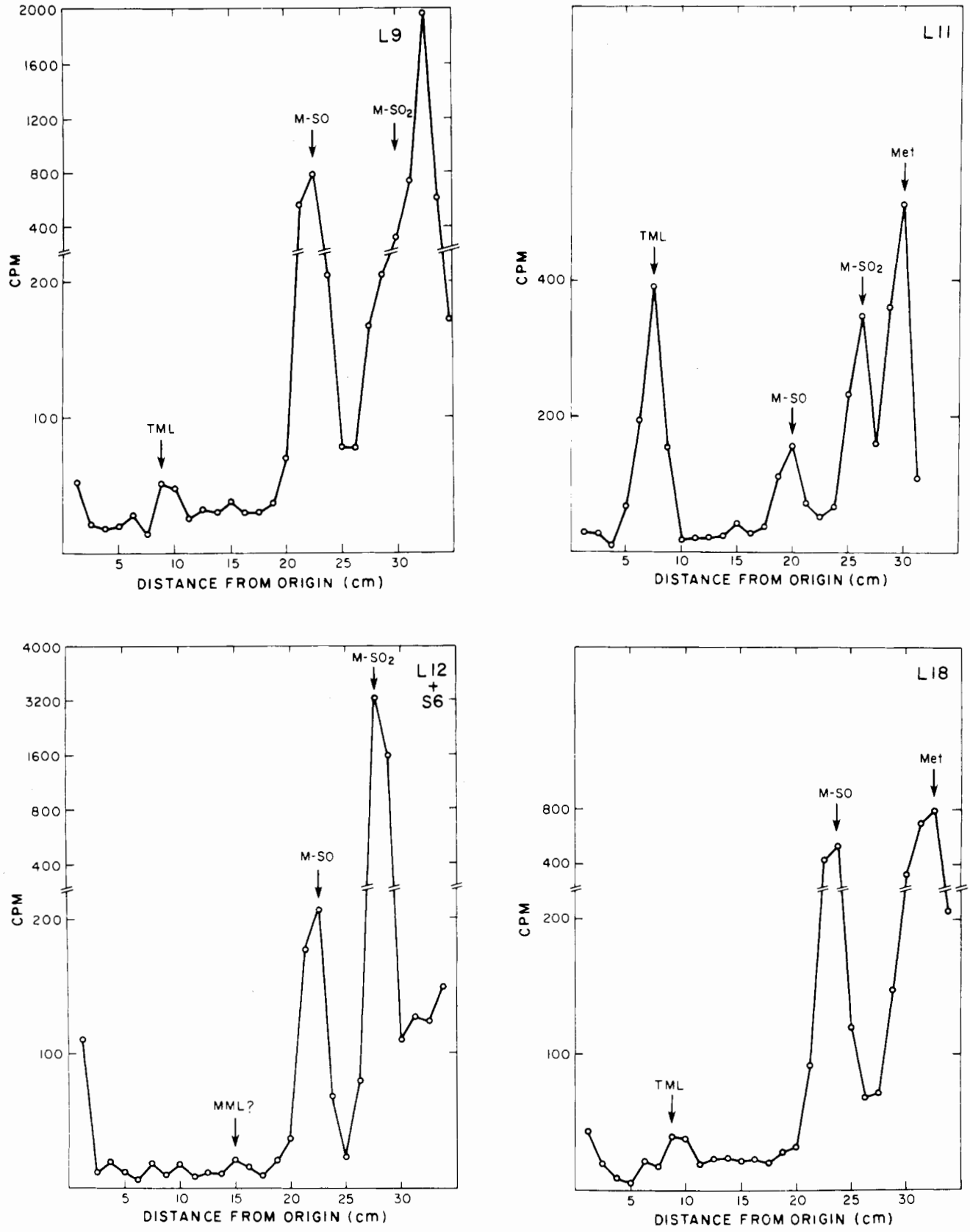

FIGURE 6: Analysis of methylated lysines deriveed from proteins L9, L11, L12 + S6, and L18 by desceding paper chromatography using solvent system (a). The conditions were the same as those described in Figure 4 except protein hydrolysates from L9, L11, L12 + S6, and L18 were used.

to contain trace amounts of ϵ-*N*-trimethyllysine. It is possible that strain difference accounts for the variations in both the extent and nature of methylation. Since 70S ribosomes were used as a starting material and protein L12 was contaminated with protein S6, the stoichiometry is uncertain for the methylated amino acids in protein L12. The low level of methylation for proteins L7 and L12 does not seem to be due to the concentration of methionine in the growth medium since the extent of methylation for most proteins was roughly the same when the concentrations of the amino

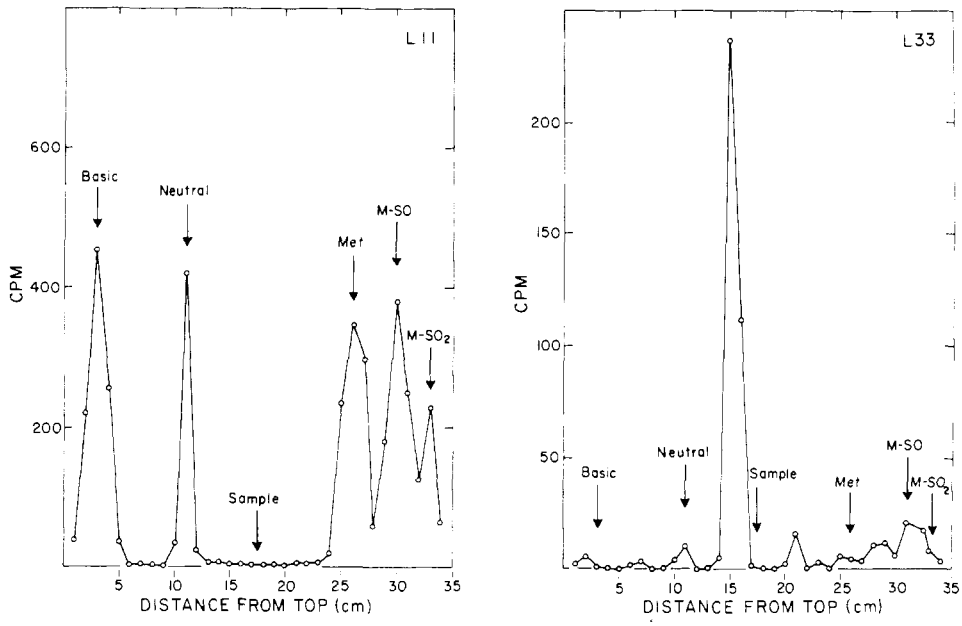

FIGURE 7: Analysis of methylated amino acids derived from proteins L11 and L33 by high-voltage paper electrophoresis in 0.05 M sodium borate buffer (pH 9.3); 20 μl of each protein hydrolysate (described under Materials and Methods) was applied to a Whatman No. 3MM paper (15 × 57 cm) at 28 cm from one end together with the following amino acids: Met, M-SO, M-SO_2, 1-MH, 3M-H, Lys, and Arg. Electrophoresis was carried out at 2000 V for 65 min. After electrophoresis the paper was dried and sprayed with ninhydrin as described in the legends of Figure 1. The paper was then cut into 1.0-cm strips and counted as described in Figure 3.

Table II: Stoichiometry of the Methylated Amino Acids in Ribosomal Proteins.

Protein	Average Mol Wt[a]	Met mol %[b]	Probable No. of Met	Methylated Amino-Acid Molecules/Protein TML	MML	sDMA	Unidentified Methylated Amino Acid(s)	Approx. Total Methyl Group[c]
L1	24,400	2.6	5	0.03	0.05	0.06	0.1	0.35
L3	25,000	2.2	4	0.07			0.2	0.4
L5	20,000	2.8	4	0.1	Trace		0.3	0.6
L7	14,500	2.6	3	0.01	0.02		0.02	0.07
L8	17,300	1.9	2–3		0.02		0.08	0.1
L9	17,300	1.9	2–3	0.01			0.07	0.1
L11	19,600	3.1	5	0.95	Trace	0.06	1.8	4.7
L12	15,000	2.3	3	Trace	0.02		0.04	0.06[d]
S6		4.5	6					
L18	15,000	1.5	2	0.01			0.1	0.13

[a] Dzionara *et al.*, 1970. [b] Kaltschmidt *et al.*, 1970. [c] The amount includes the unidentified methylated neutral amino acid(s) detected by high-voltage paper electrophoresis in 0.05 M sodium borate buffer (pH 9.3). The nature of the unidentified methylated amino acid(s) is unknown. We have also detected a very small amount of the unidentified methylated amino acid(s) from the [^{14}C-*methyl*]methionine hydrolysate alone (see text). [d] Assuming proteins L12 and S6 are present in equal amounts.

acids were increased to 30 μg/ml (unpublished observations). The only exception to this is the amount of ϵ-*N*-trimethyllysine in protein L11. In a total of six separate experiments we have obtained values of ϵ-*N*-trimethyllysine in protein L11 ranging from 0.8 to 1.1 molecules/molecule of protein from cells grown with 7–30 μg/ml of methionine. Thus it is possible that the variation in the extent of methylation for this protein is due to the growth conditions. Independently, Alix and Hayes (1974) have also observed the presence of nearly one molecule of ϵ-*N*-trimethyllysine in protein L11 and the methylation of proteins L3 and L5. Protein L11 has been claimed to be involved in the peptidyl transferase reaction during protein synthesis (Nierhaus and Montejo, 1973). The ϵ-*N*-trimethyllysine residue in protein L11 could be important for the peptidyl transferase reaction. It is uncertain whether the trimethylation is localized in only one lysine residue or whether more than one lysine residue is involved.

Perhaps the most interesting of all the methylated proteins is L1 which contains three to four different methylated amino acids in approximately equal amounts (~0.05 molecule/protein). The function of this protein in the 50S subunit is currently unknown. It is also unknown whether every L1 protein molecule contains the same amount of these methylated amino acids. Nor do we know whether this is related to the heterogeneity of the 50S ribosomal subunits. Proteins L5, L11, and L18 have been suggested to be involved in the elongation factor G-dependent GDP binding to the 50S subunit (Maassen and Möller, 1974), and proteins L7 and L12 have previously been implicated in

the translocation reaction (Terhorst *et al.*, 1972). The methyl groups in these proteins may play some role in the functioning of the above mentioned reactions. Since the level of methylation for several proteins (*e.g.*, L8, L9, and L18) was low, we have not completely ruled out the possibility that they may be due to the contamination of other methylated nonribosomal proteins.

It should be pointed out that the sensitivity of the double labeling experiment (Table I) is dependent upon the number of methionine residues in a protein. The lower the number of methionine residues in a protein, the more sensitive the procedure in detecting the methylation of proteins. The reason for the high $^3H/^{14}C$ ratio for protein L33 (Table I) is due to the fact that protein L33 is devoid of methionine (Kaltschmidt *et al.*, 1970). This is probably also true for the rather high $^3H/^{14}C$ ratios observed for both proteins (L8 + L9) and L18, even though they contain only 0.1 methyl group/molecule of protein. Both proteins (L8 + L9) and L18 contain about two methionine residues. Although proteins L1, L3, and L5 are more heavily methylated when compared to other proteins (*e.g.*, proteins L9 and L18), they do not give rise to high $^3H/^{14}C$ ratios because they contain at least four methionine residues in each protein.

Because of the high pH (pH 8.6) of the first dimension run of the two-dimensional polyacrylamide gel electrophoresis, the alkaline-labile methyl esters of glutamic and aspartic acids, if present, would not be detected by our present procedure. In all the studies we have assumed that the methyl donor is *S*-adenosylmethionine. Recently, methyltetrahydrofolates have been reported to be efficient methyl donors in the synthesis of biogenic amines (Banerjee and Snyder, 1973). It is unknown whether such a methylating system is operating in *E. coli*. Since 5-methyltetrafolate is used to regenerate methionine from homocysteine, it would be difficult to determine if 5-methyltetrafolate is directly involved in the methylation of proteins in *E. coli*.

We have also studied the methylation of ribosomal proteins *in vitro* from 50S particles derived from an *E. coli rel*$^-$ *met*$^-$ strain after starvation for methionine (Chang and Chang, 1974). Protein L11 and to a lesser extent proteins L1, L3, and L5 were methylated. The level of *in vitro* methylation agreed quite well with our present *in vivo* methylation studies (Table II). This indicates that proteins L1, L3, L5, and L11 are the major methylated proteins in the cell and they may be very important in the functioning of the 50S ribosomal subunit. Proteins L7, L8, L9, L12, and L18 from the *E. coli rel*$^-$ *met*$^-$ strain were not methylated *in vitro*. Since these proteins contain only small amounts of methylated amino acids (Table II), it appears that unless one uses a very sensitive procedure, methylation will not be detected *in vitro*.

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