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Purification and Characterization of a tRNA Methylase from *Salmonella typhimurium*

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A tRNA methylase, in which *supK* strains of *Salmonella typhimurium* are deficient, was purified from strain LT2 and characterized. Column chromatography of protein extracts from wild-type cells on phosphocellulose, diethylaminoethyl-Sephadex A-50, and hydroxylapatite resulted in an enzyme that was estimated to be about 50% pure. tRNA from *S. typhimurium* which had been incubated at pH 9.0 served as a substrate for this methylase. The enzyme has a molecular weight of about 50,000 as estimated by gel chromatography and by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. The optimal assay conditions, as well as the kinetics and stability of the enzyme, were studied. As with other tRNA-methylating enzymes, *S*-adenosylhomocysteine is a potent inhibitor.

The *supK* strains of *Salmonella typhimurium* have suppressor activity for the nonsense triplet UGA (20) and weak suppressor activity for certain frameshift mutations (2). The recessive nature of the *supK* mutation for suppressor activity indicates that a tRNA gene is not altered but that these strains might be deficient in a function modifying tRNA (20). The results of Reeves and Roth (21) indicate that the suppressor strains might be deficient in a specific tRNA methylase activity. First, the tRNA from such strains is methyl deficient. Second, with methyl-deficient tRNA as the substrate, a specific tRNA methylating activity from *supK* strains is reduced to about one-third the level found in wild-type cells.

The methylase is specific for only a few species of tRNA. We have identified two species that serve as substrates for this methylase, an alanine and a serine tRNA, and there appears to be a third, as yet unidentified, tRNA substrate (18). A possible model to account for the suppressor activity in *supK* strains is that these tRNA's are undermethylated due to a deficiency in this specific methylase. These undermethylated tRNA's miscode, and one may recognize the UGA codon as a sense codon. It has been suggested that these undermethylated tRNA's also do not function in their normal codon recognition, since complete loss of the *supK* function appears to be a lethal event (20, 21). Complete loss of the methylating activity would lead to tRNA that is totally deficient in this methyl group, and these tRNA's may not function in protein synthesis.

To demonstrate that a deficiency in this methylating enzyme results in suppressor activity, we have investigated both the enzyme itself and the products of methylation (18). This report describes the purification and properties of the methylating enzyme in which *supK* strains are deficient.

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MATERIALS AND METHODS

Materials. Most of the biochemicals used in this work were obtained, in the purest grade available, from Sigma Chemical Co. Sephadex G-100 and A-50 were obtained from Pharmacia Fine Chemicals, Inc. All gel electrophoresis reagents and the electrophoresis apparatus were purchased from Bio-Rad Laboratories. The hydroxylapatite (Bio-Gel HT) was also from Bio-Rad. The cellulose ion-exchange materials were obtained from Reeve Angel. The source of the radioactive *S*-adenosyl-L-methionine (SAM) was Amersham Corp.

Preparation of tRNA. tRNA used as the substrate for the tRNA methylase was prepared from *S. typhimurium* LT2 (obtained from J. R. Roth) in two ways, differing mainly in pH. To prepare tRNA having a high level of methyl group-accepting activity, the following procedure was used. Bacteria from 40 liters of E medium (27) were harvested in late logarithmic growth, and the cells (ca. 60 g [wet weight]) were suspended in 200 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5)-0.01 M $MgCl_2$. The tRNA was extracted by sonic treatment in the presence of 100 ml of 88% phenol for 10 min

with the large probe of a Sonifier-Cell Disruptor (model W140), keeping the temperature between 0 and 5°C. After 2 min of vigorous shaking, the aqueous phase was separated by centrifugation. Another 200 ml of cold Tris buffer was added to the phenolic phase, and after the solution was shaken for 5 min the second aqueous phase was separated by centrifugation. The tRNA was recovered from the combined aqueous phase by diethylaminoethyl (DEAE)-cellulose column chromatography as described by Riddle and Roth (22). The resulting tRNA was further purified by gel filtration on a Sephadex G-100 column (2.5 by 90 cm) equilibrated with the above-mentioned Tris-$MgCl_2$ buffer. This step removes as much as 40% of the high-molecular-weight RNA. The tRNA pool (measured by its absorbance at both 260 and 335 nm [A_{260} and A_{335}, respectively]) was precipitated with 2 volumes of 95% ethanol and stored at −20°C in 10 mM Tris-hydrochloride (pH 7.5)-10 mM $MgCl_2$, usually at 5 mg/ml. This material, prepared and stored frozen at pH 7.5, had a methyl group acceptance level of between 40 and 150 pmol/A_{260} unit (55 μg) of tRNA with the purified enzyme. This value slowly increases to 150 pmol/A_{260} unit of tRNA upon storage at −20°C (18). Incubation of the tRNA at pH 9.0 (0.5 M Tris-hydrochloride, pH 9.0; 10 mM $MgCl_2$) for 1 h followed by ethanol precipitation results in tRNA capable of accepting 150 pmol of methyl group per A_{260} unit.

To preserve the methyl groups on the tRNA added in vivo, a second procedure was used. After the cells were harvested, they were washed and suspended in 200 ml of 100 mM Tris-acetic acid (pH 7.0)-10 mM $MgCl_2$. The phenol extractions were then carried out as outlined above, except for the difference in buffers. The aqueous phases were immediately adjusted to pH 5.0 with acetic acid after separation from the phenol. The DEAE-cellulose and Sephadex G-100 column chromatography steps were performed in the same way as for the first procedure, except that the buffer was 0.1 M sodium acetate (pH 5.0)-10 mM $MgCl_2$. All steps in this second procedure were performed at 0 to 5°C. This tRNA preparation, stored at pH 5.0, has a methyl acceptance of less than 10 pmol/A_{260} unit of tRNA; this value does not increase upon storage.

Undermethylated tRNA was prepared from *S. typhimurium* TR 1350 (*rel met*) as described by Reeves and Roth (21).

Assay of tRNA methylase. tRNA methylase was assayed as described by Reeves and Roth (21), except that the buffer and pH were changed to correspond to optimal assay conditions. The assay was carried out at 37°C usually in a 250-μl solution (total volume) consisting of: 40 mM potassium phosphate (pH 7.5), 4 mM $MgCl_2$, 2 mM L-methionine, 20 μM [*methyl*-^{14}C]-SAM (50 Ci/mol), 0.8 mg of tRNA, and approximately 0.2 μg of the purified enzyme. At various times, 50-μl portions were removed and applied to 2.5-cm Whatman 3MM filter disks. The disks were washed once in cold 10% trichloroacetic acid for 20 min (10 ml per filter), twice in cold 5% trichloroacetic acid (10 min each, 10 ml per filter), once in ethanol-ether (50:50, vol/vol), and finally in ether. After drying for 15 min at 60°C, the filters were placed in scintillation vials with 5 ml of Aquasol II (New England Nuclear Corp.), and the radioactivity was determined in a Packard scintillation spectrometer.

For measuring the methylase activity from column effluents, the assay was usually performed in a total volume of 62.5 μl, and one 50-μl portion was taken at 40 min for the determination of acid-insoluble radioactivity.

Purification of the *supK* tRNA methylase. We have designated this methylase the *supK* methylase since we have not identified the methylated nucleoside product of the enzyme (18) and since this enzyme appears to be the *supK* gene product (20, 21). Details of the purification procedure are presented here and outlined in Results. At all times the enzyme was kept between 0 and 4°C. Cells (*S. typhimurium* LT2, 60 g [wet weight]) were harvested during late logarithmic growth in E medium. The cells were washed with 150 ml of buffer A (0.1 M triethanolamine-hydrochloride, pH 7.8; 1 mM sodium ethylenediaminetetraacetate [EDTA]; 0.4 mM dithioerythritol; 10% glycerol [vol/vol]), followed by centrifugation. The cells were suspended in 200 ml of buffer A and sonically disrupted in short pulses for 6 min. The cellular debris was removed by centrifugation at 30,000 × *g* for 1 h. Ribosomes were removed by centrifugation of the supernatant for 2 h at 106,000 × *g*. The clear, yellowish supernatant (fraction I, see Table 1) was treated with 20 mg of streptomycin sulfate per ml, and the white precipitate was removed by centrifugation at 30,000 × *g* for 30 min. The supernatant (fraction II) was dialyzed against two changes of buffer A adjusted to pH 8.8. It was applied to a phosphocellulose column (2.5 by 37 cm) equilibrated with buffer A (pH 8.8). The column was washed with 300 ml of the same buffer, followed by 250 ml of buffer B (50 mM potassium phosphate, pH 7.5; 1 mM sodium EDTA; 0.4 mM dithioerythritol; 10% glycerol [vol/vol]). Protein that bound to the column was eluted with a 1,400-ml linear NaCl gradient (0 to 0.70 M) in buffer B. The *supK* methylase activity was pooled, dialyzed (buffer B), and concentrated by ultrafiltration with an Amicon PM 10 membrane (fraction III).

Fraction III was brought to 0.08 M NaCl in buffer B and applied to a DEAE-Sephadex A-50 column (1.2 by 30 cm) equilibrated with the same buffer. The column was washed with 80 ml of the same buffer, followed by a 300-ml linear gradient (0.08 to 0.55 M NaCl) in buffer B. Fractions containing methylase activity were pooled (fraction IV) and dialyzed against buffer C (10 mM potassium phosphate, pH 7.5; 0.4 mM dithioerythritol; 10% [vol/vol] glycerol). The dialyzed solution was applied to a column (1.6 by 7 cm) of hydroxylapatite equilibrated with buffer C. The methylase activity was eluted with a step gradient with increasing concentrations of potassium phosphate (pH 7.5), containing the other components in buffer C: 30 ml of 0.01 M potassium phosphate; 33 ml of 0.07 M potassium phosphate; 33 ml of 0.10 M potassium phosphate; 36 ml of 0.13 M potassium phosphate; and 40 ml of 0.16 M potassium phosphate. The enzyme activity, eluting at 0.13 M potassium phosphate, was pooled (fraction V). The fraction V enzyme was estimated to be 50% pure by polyacrylamide gel electrophoresis, and most of the following characteri-

zations were performed with this enzyme fraction.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out as described by Davis (4), using a Tris-Tricine buffer system (pH 8.2). The upper buffer contained 3.62 g of Tris and 6.02 g of Tricine per liter, and the lower buffer consisted of 0.1 M Tris-hydrochloride (pH 8.2). The resolving gel (8% acrylamide) was formed by combining 10 ml of 30% acrylamide and 0.8% *N,N'*-methylenebisacrylamide, 20 ml of 0.1 M Tris-hydrochloride (pH 7.5), 1.8 ml of 0.5% *N,N,N',N'*-tetramethylethylenediamine, and 0.55 ml of 1.6% ammonium persulfate. Electrophoresis was normally carried out at 4°C for about 2 h at 1.5 to 2.0 mA per tube. Gels were stained for 4 h at 37°C with 0.5% Coomassie brilliant blue in methanol-acetic acid-water (5:1:5, vol/vol/vol) and destained by soaking at room temperature in 10% acetic acid-5% methanol. Densitometer tracings were made with an Isco type 6 gel scanner.

SDS-polyacrylamide gels. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in 10% gels was performed by the method of Weber and Osborn (25), except that gels were prepared with a double cross-linker concentration. Sample preparation was accomplished by dissolving the protein in 0.05 M sodium phosphate (pH 6.8) containing 1% SDS and 1% 2-mercaptoethanol and heating to 100°C for 2 min. For molecular weight determinations, electrophoresis of six standard proteins was performed in parallel: ribonuclease A (molecular weight, 13,700), lysozyme (molecular weight, 14,300), lactic dehydrogenase (molecular weight, 36,000), aldolase (molecular weight, 39,500), ovalbumin (molecular weight, 45,000), and pyruvate kinase (molecular weight, 57,000). Gels were stained and destained as described above.

Gel filtration. The molecular weight of the *supK* tRNA methylase was determined by gel filtration on a Sephadex G-100 column (1 by 90 cm) at 4°C. Fraction III or IV enzyme was applied to the column and eluted with buffer B at 0.3 ml/min. Fractions (1.0 ml) were assayed for methylase activity as described above. Four protein standards, ribonuclease A, chymotrypsinogen (molecular weight, 25,000), ovalbumin, and bovine serum albumin (molecular weight, 68,500) plus blue dextran 2000 (Pharmacia), were chromatographed on the same column, and their elution positions were monitored by measuring the A_{280}. The data were analyzed by the method of Whitiker (26), plotting peak elution volume/void volume (V_e/V_0) versus log molecular weight.

Protein assay. Protein concentrations were determined by the method of Lowry et al. (14), with crystalline bovine serum albumin as a standard. Protein from column effluents was monitored by measuring the A_{280}.

RESULTS

Purification of the tRNA methylase. Purification of the *supK* tRNA methylase can be followed without the interference of other tRNA methylase activities because a specific substrate tRNA can be obtained for the *supK* methylase. The methyl group added by this enzyme is alkali labile and can be hydrolyzed to a volatile product, probably methanol (18). The methyl group can then be returned enzymatically by using [*methyl*-^{14}C]SAM as the methyl donor. The tRNA substrate normally used for the *supK* methylase assay is extracted from wild-type *S. typhimurium* and incubated at pH 9.0 for 1 h (see Materials and Methods). However, even tRNA which is extracted by normal phenol extraction procedures at pH 7.5 and not incubated at high pH has some methyl acceptor activity due to slow hydrolysis of the methyl group during extraction (18).

The methylase was purified to near homogeneity by three column chromatographic steps. Table 1 gives a summary of the purification scheme. Results of the first two steps are shown in Fig. 1. The profiles for total protein (A_{280}) and for total tRNA methylase activity are included with the profiles for the *supK* methylase activity. The total tRNA methylase activity in Fig. 1 was detected by using undermethylated tRNA as the substrate; this was obtained from *rel met* bacteria after methionine starvation. The phosphocellulose column step (Fig. 1A) achieved about a 20-fold purification of the *supK* methylase, but the pooled enzyme (fraction III, Table 1) was still contaminated with at least one other tRNA methylase activity and with some ribonuclease activity that degraded the tRNA and interfered with the methylase assay. However, after the DEAE-Sephadex column step (Fig. 1B) the *supK* methylase was essentially free from these two other activities (fraction IV, Table 1). Polyacrylamide gel electrophoresis of the enzyme pool from fraction IV under nondenaturing conditions revealed several protein bands (Fig. 2A).

The methylase was purified to near homogeneity by chromatography on hydroxylapatite. The methylase activity eluted with 0.13 M potassium phosphate (pH 7.4) in a step gradient of potassium phosphate (fraction V, Table 1). This enzyme fraction gave one major band upon polyacrylamide gel electrophoresis (Fig. 2B). In separate, parallel gel runs with this fraction V enzyme, enzymatic activity corresponded in position to this major protein band (Fig. 3A and 3B). This enzyme fraction also gave one major band upon SDS-polyacrylamide gel electrophoresis (Fig. 4A). Fraction V was used for most of the following experiments on the properties of the enzyme.

In crude extracts, the *supK* tRNA methylase was relatively unstable. It was unstable in comparison with other tRNA methylase activities found in *S. typhimurium* and much more unstable than aminoacyl-tRNA synthetases. Glycerol, other proteins, mercaptans, tRNA, and SAM did

TABLE 1. *Summary of purification of the supK tRNA methylase*

Enzyme fraction	Protein (mg)	U[a]	Sp act (U/mg)	Yield (%)	Purification
I. Crude extract	5,200	40,000	7.7	100	1.0
II. Streptomycin sulfate	4,000	40,000	10	100	1.3
III. Phosphocellulose	68	12,000	180	29	23
IV. DEAE-sephadex	4.8	9,100	1,900	23	250
V. Hydroxylapatite	0.25	3,500	14,000	8.8	1,800

[a] One unit of activity is equivalent to the formation of 1 pmol of methyl-tRNA in 1 min at 37°C.

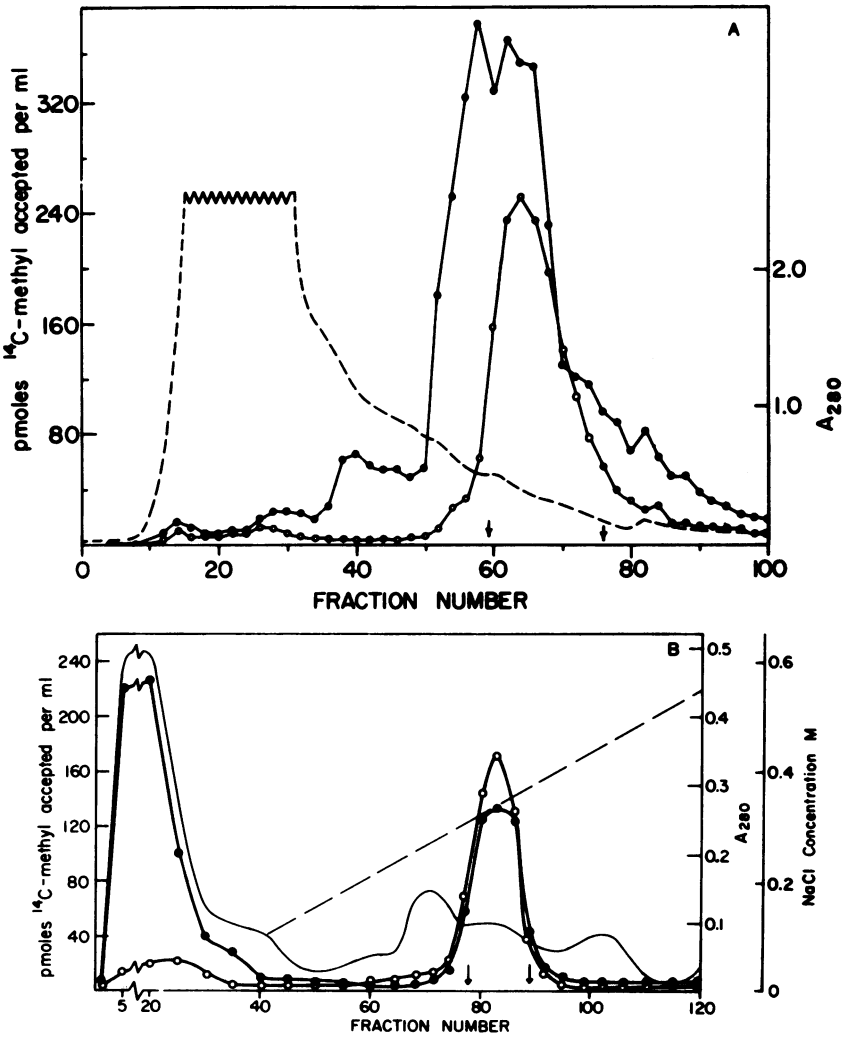

FIG. 1. *Column chromatography of the supK tRNA methylase. (A) Chromatography of fraction II (Table 1) crude enzyme on a phosphocellulose column (2.5 by 37 cm). At fraction 40, a 1,400-ml 0 to 0.7 M NaCl gradient (in buffer B) was started (see text). Fractions contained 15 ml. The supK methylase activity in fractions 59 to 76 was pooled. Symbols: — — —, A_{280}; ●, total tRNA methylase activity with methyl-deficient tRNA as the substrate; ○, supK methylase activity. (B) Chromatography of fraction III (Table 1) enzyme on a DEAE-Sephadex A-50 column (1.2 by 30 cm). At fraction 40, a 300-ml 0.08 to 0.55 M NaCl linear gradient in buffer B was started (see text). Fractions contained 3.0 ml. The tRNA methylase activity in fractions 76 to 90 was pooled. Symbols: — — —, NaCl concentration (M); ———, A_{280}; ●, total tRNA methylase activity; ○, supK tRNA methylase activity.*

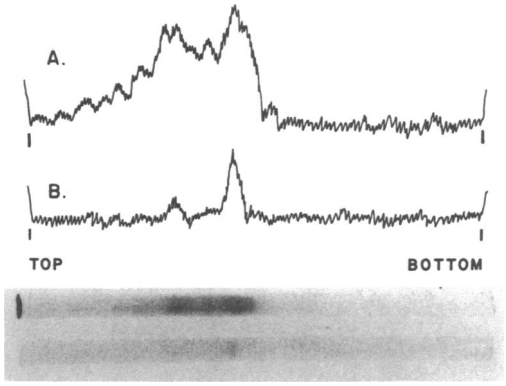

FIG. 2. *Polyacrylamide gel electrophoresis of fraction IV and fraction V methylase preparations. Electrophoresis was carried out by the method of Davis (4) at 4°C (see text). Photographs were made of the gels (shown below), and the negatives were scanned with a Joyce-Loebl densitometer (A and B above). (A) Fraction IV enzyme, 10 μg of protein. (B) Fraction V enzyme, 2 μg of protein. The major band corresponds to 52% of the total protein of this gel.*

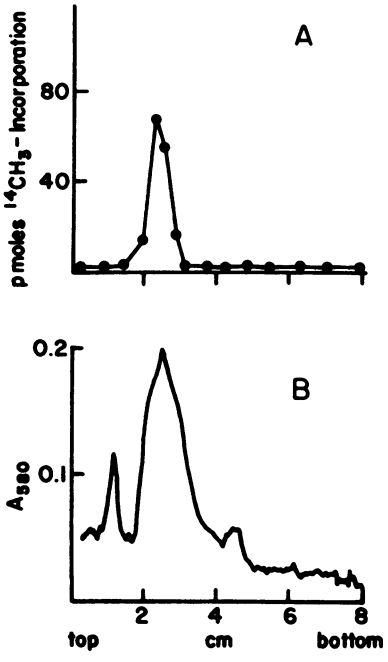

FIG. 3. *Polyacrylamide gel electrophoresis of fraction V enzyme. Approximately 5 μg of protein from fraction V was applied to each of two gels, which were subjected to electrophoresis in parallel by the method of Davis (4) (see text). (A) One gel was sliced into 3-mm slices. Protein was eluted from each slice with 0.20 ml of 50 mM potassium phosphate (pH 7.5)-4 mM dithioerythritol at 4°C for 60 min. Portions were immediately assayed for tRNA methylase activity. (B) The second gel was stained and scanned in an Isco type 6 gel scanner.*

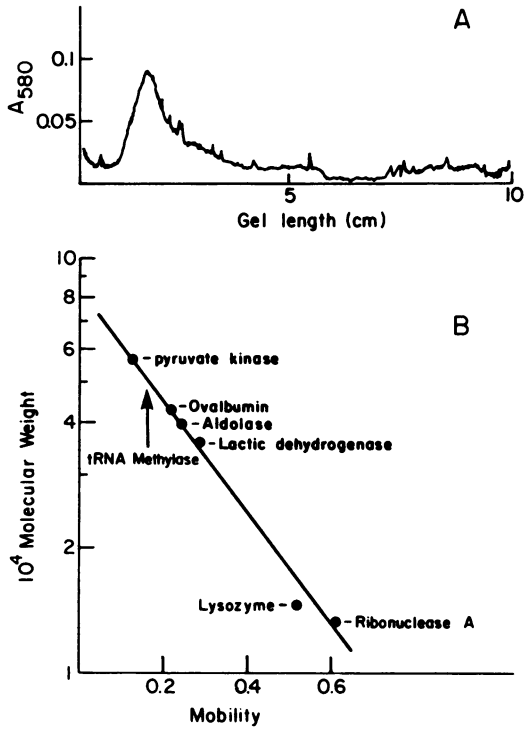

FIG. 4. *Molecular weight determination on the supK tRNA methylase by SDS-polyacrylamide gel electrophoresis. (A) Approximately 5 μg of fraction V enzyme (Table 1) was run on a 10-cm gel as described in the text. The stained gel was scanned at 580 nm in an Isco gel scanner. (B) Molecular weight estimation of the tRNA methylase by SDS-polyacrylamide gel electrophoresis. The mobility of the tRNA methylase is plotted with mobilities of five protein standards. The data are plotted by the method of Weber and Osborn (25).*

not stabilize the enzyme to any appreciable extent. The enzyme lost 30% of its activity per day at 0°C in crude extracts (fractions I or II), which accounts for the poor yield of enzyme seen in fraction III. After the phosphocellulose column step (fractions III and IV) the enzyme could be stored for several months at 0°C with no detectable loss in activity. Chromatography of crude enzyme extracts on DEAE-cellulose or DEAE-Sephadex as a first column step did not stabilize the enzyme. The fraction V enzyme, even though nearly homogeneous, again became unstable and lost about 25% of its activity per week when stored at 0°C. There was also a pronounced loss of activity upon dilution of the purified enzyme, which was not seen with fractions III and IV. The purified enzyme was also much more heat labile than the fraction IV enzyme. This instability was again insensitive to the addition of protein (bovine serum albumin), glycerol, mer-

captans, and substrates. One possible explanation for this instability is that a specific protein from *S. typhimurium* is required for stabilization of the methylase and is removed on hydroxylapatite.

Molecular weight of the *supK* methylase. The molecular weight of the *supK* methylase was determined by gel filtration on Sephadex G-100 columns and by SDS-polyacrylamide gel electrophoresis using protein standards in parallel runs (see Materials and Methods). The molecular weight of the active protein by gel filtration was found to be 50,000 ± 3,000. By its mobility upon SDS-polyacrylamide gel electrophoresis, the protein from fraction V was found to have a molecular weight of 52,000 ± 3,000 (Fig. 4A and B). Because of the instability of the fraction V enzyme, no enzymatic activity of the methylase could be recovered from Sephadex G-100 columns in several attempts. The fraction III and IV enzymes, therefore, were used for the molecular weight determination by gel filtration, although, even with these more stable fractions, more than 40% of the initial activity was not recovered. Taken together these two determinations indicate that the *supK* methylase is a single polypeptide chain with a molecular weight of about 50,000.

Optimal assay conditions. Figure 5 and Table 2 give the results of the studies of pH and salt effects on enzyme activity. The pH optimum was about 7.5 in 40 mM potassium phosphate buffer (Fig. 5A). This value is low compared with that of other bacterial tRNA methylases studied which have optima between pH 7.5 and 9.0 (9). This lower pH optimum may be due in part to the lability of the added methyl group at higher pH values (18). It is also apparent from Fig. 5A that phosphate is a better buffer for the enzyme than triethanolamine. Potassium phosphate had a stimulatory effect on the enzyme (Fig. 5B; Table 2) which is maximal at 40 mM, and NaCl and KCl also had a slight stimulatory effect even at concentrations as high as 160 mM. Magnesium chloride had little effect on enzyme activity, and in fact divalent cations do not appear to be required for activity since 20 mM EDTA had no inhibitory effect. Even at 40 mM EDTA the enzyme retained 60% of its activity. These levels of EDTA at the ionic strength used in the assay should result in some disruption of the tertiary structure of the tRNA substrate (19). These results indicate that the overall conformation of the tRNA may not be crucial for methylase recognition, although local conformations may still be required.

Methylase activity on various RNAs. The *supK* methylase appears to be specific for bacterial tRNA. The data in Table 3 indicate that the enzyme will methylate the tRNA of *S. typhimurium* and *Escherichia coli* B. The tRNA must first be stripped of in vivo-added methyl groups before it acts as an efficient substrate (18). This can be accomplished by storing the tRNA at pH 7.5 for a long period of time or by incubation at pH 9.0 for 1 h at 37°C. So far, we have found no other RNA that accepts methyl groups with the purified enzyme. Yeast total RNA or tRNA and bacterial rRNA were inactive as substrates (Table 3).

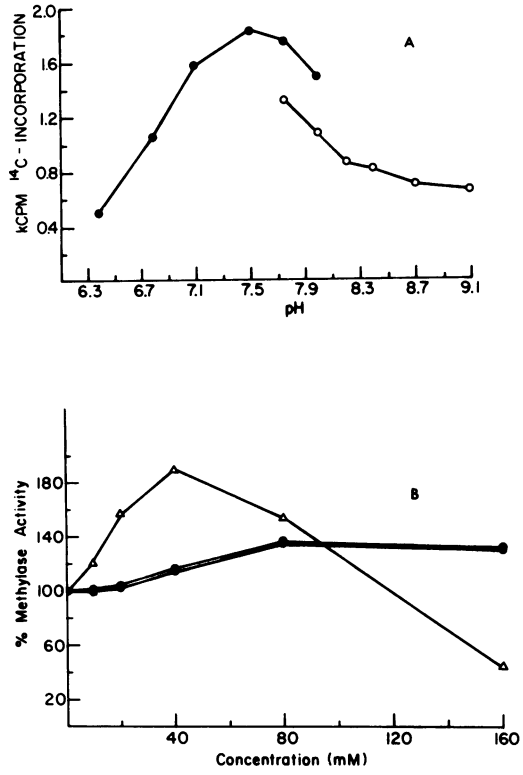

FIG. 5. *Effect of pH and salt concentrations on tRNA methylase activity. (A) The effect of pH, using 40 mM potassium phosphate (●) or 40 mM triethanolamine-hydrochloride (○), on tRNA methylase activity. Fraction V enzyme was assayed as described in the text, except that the buffer and pH were as indicated in the figure. (B) Effect of various salt concentrations on tRNA methylase activity. The enzyme was assayed as described in the text, except that the buffer was 40 mM triethanolamine-hydrochloride (pH 7.6). Symbols: △, potassium phosphate (pH 7.6); ●, NaCl; ○, KCl.*

Saturation of the tRNA with methyl groups. The tRNA methylase acts on specific sites on the tRNA, and, at high ratios of enzyme to tRNA and with long reaction times, the tRNA becomes saturated with methyl groups (9). Fig-

ure 6 shows the results of such an in vitro saturation experiment with the purified enzyme. The saturation level is due to the amount of tRNA added to the reaction and not to the loss of SAM or inactivation of the enzyme. The addition of these two components at 92 min had no effect on the level of methylation. Only the addition of more tRNA raised the plateau level, and the new level gave the same value for picomoles of methyl group accepted per total tRNA added as the lower level. Using tRNA that had been incubated at pH 9.0 for 1 h, we obtained a value of approximately 150 pmol of methyl group accepted per A_{260} unit of tRNA (1,700 pmol). If a correction is made for the rate of chemical hydrolysis of the methyl groups added to the tRNA during the assay (18), there are approximately 170 pmol of methyl-accepting sites per A_{260} unit of tRNA. Thus, if there is one site per tRNA, 1 molecule in 10 is accepting a methyl group.

TABLE 2. *Effect of salts on tRNA methylase activity*[a]

Addition (mM)	$^{14}CH_3$ incorporation (%)
None	100
NaCl (80)	133
KCl (80)	135
KH_2PO_4 (40)	195
$MgCl_2$ (10)	125
EDTA (20)	110
EDTA (40)	60

[a] The enzyme was assayed as described in the text, except that the buffer was triethanolamine-hydrochloride (pH 7.6). The final concentration of $MgCl_2$ was 4 mM except where indicated otherwise.

TABLE 3. *Methylation levels of various RNAs*[a]

RNA	^{14}C-*methyl* incorporated (pmol/A_{260})
tRNA, *S. typhimurium* LT2	8.0
tRNA, *S. typhimurium* LT2 (pH 9 incubated)	150.0
tRNA, *E. coli* B	121.0[b]
tRNA, yeast (brewers') (pH 9 incubated)	0.3
rRNA, *S. typhimurium* LT2 (pH 9 incubated)	0.7
Total RNA, yeast (pH 9 incubated)	0.0

[a] The methylation level for each RNA was determined with fraction VI enzyme, using the assay conditions described in the text.

[b] The level of methylation of *E. coli* B tRNA may be higher. This substrate was not incubated at pH 9.0. The methylated product in *E. coli* B tRNA is alkali labile, with a rate of hydrolysis very similar to that of the methylated product in *S. typhimurium* tRNA (18).

Temperature stability of the enzyme. The enzyme lost 50% of its activity in about 5 min at 50°C (Fig. 7). Neither of the two substrates, SAM or tRNA, was able to protect the enzyme against heat inactivation. The presence of SAM even increased the heat lability of the enzyme. We did not determine the heat stability of the enzyme under assay conditions, but we did observe linear incorporation of radioactive methyl groups for up to 60 min.

Effect of sulfhydryl reagents on methylase activity. The data in Table 4 give the effects of various sulfhydryl reagents on the tRNA methylase activity. Incubation of the enzyme with 0.01 mM *p*-hydroxymercuribenzoate resulted in over 95% inhibition of enzyme activity. This loss of activity was prevented by relatively high concentrations of mercaptoethanol. The two substrates, SAM and tRNA, singly or together did not appear to offer any protection against the inhibitory action of *p*-hydroxymercuribenzoate, just as in the case of heat inactivation presented above. Other sulfhydryl reagents, such as *N*-ethylmaleimide and iodoacetamide, also inhibited the enzyme, but not as effectively as *p*-hydroxymercuribenzoate. Other bacterial tRNA methylases are also inhibited by sulfhydryl reagents (9).

Effect of *S*-adenosylhomocysteine on methylase activity. As with other methylating enzymes that use SAM as a methyl donor, the *supK* tRNA methylase is sensitive to inhibition by *S*-adenosylhomocysteine, the product of the methylase reaction (7–9). Figure 8 shows that 50% inhibition of methylase activity occurred at about 7 μM *S*-adenosylhomocysteine (with 20 μM SAM in the assay). Data not shown here indicate *S*-adenosylhomocysteine is a competitive inhibitor of SAM for the enzyme. Methionine at 40 and 400 μM and adenosine and other nucleosides at 40 μM had no inhibitory effect on the methylase.

Effect of substrate concentrations on methylase activity. The initial rate of tRNA methylation by the purified enzyme was determined to be a function of tRNA and SAM concentrations. These data were treated by the method of Lineweaver and Burk (13). The observed K_m for SAM was 2.0 μM. The K_m for methyl-accepting tRNA was approximately 0.35 μM. This value was calculated from the observed K_m of 2.0 A_{260} units per ml of crude tRNA, assuming 170 pmol of methyl-accepting tRNA species per A_{260} unit of tRNA. Both K_m values may be slightly high (about 10%) due to problems in reaching enzyme-saturating concentrations for the nonvariable substrate in each assay. At least 10 times the K_m value for each nonvar-

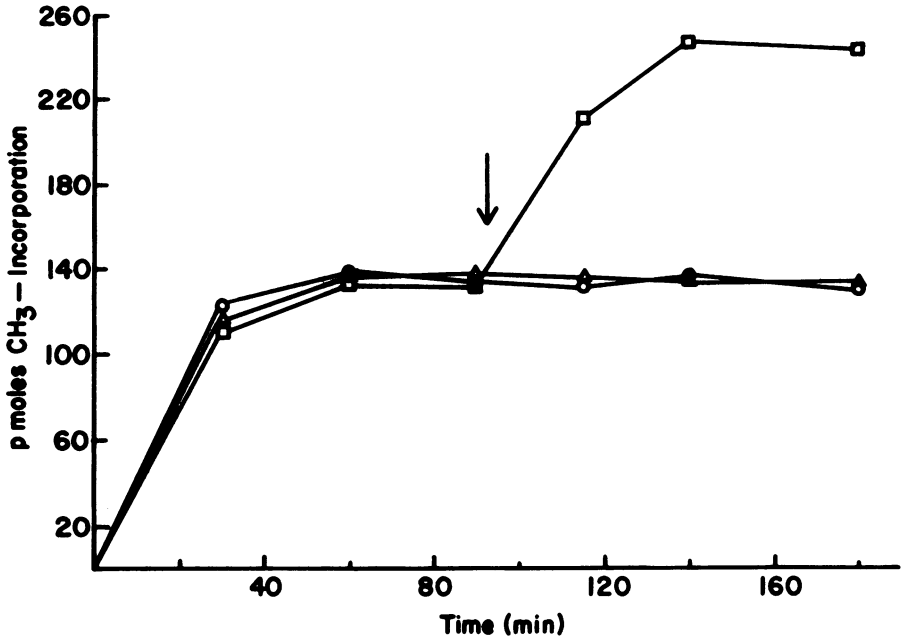

FIG. 6. *Saturation of tRNA with methyl groups. Fraction V enzyme (4 μg) was used to methylate tRNA (7.2 A_{260} units) that had been incubated at pH 9.0 for 1 h. Three assays were carried out in final volumes of 400 μl so that seven 50-μl samples could be removed at various times (see text). At 92 min was added 3.6 A_{260} units of tRNA (□), 4 nmol of [methyl-^{14}C]SAM (△), or 2 μg of fresh fraction V enzyme (○). Blank assays with heat-treated enzyme were also performed. Corrected methyl group incorporation (picomoles) into acid-insoluble material per 50-μl portion is plotted against the time of the assay.*

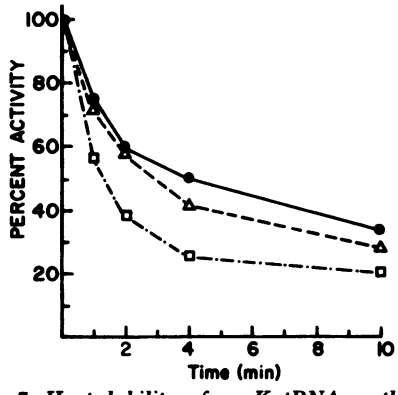

FIG. 7. *Heat lability of supK tRNA methylase. Fraction V enzyme (Table 1) was preincubated at 50°C for the times indicated and then assayed as described in the text. Symbols: ●, preincubation of enzyme alone; △, preincubation of enzyme plus 0.5 mg of tRNA per ml; □, preincubation of enzyme plus 10 μM SAM.*

TABLE 4. *Effect of sulfhydryl reagents on tRNA methylase*[a]

Addition(s) (mM)	$^{14}CH_3$ incorporation (%)
None	100
HO-HgBzO[b] (0.01)	3
HO-HgBzO (0.01) and 2-mercaptoethanol (1.0)	105
HO-HgBzO (0.01) and SAM (0.01)	3
HO-HgBzO (0.01) and tRNA (10 A_{260}/ml)	1
HO-HgBzO (0.01), SAM (0.01), and tRNA (10 A_{260}/ml)	2
N-ethylmaleimide (0.1)	35
N-ethylmaleimide (1.0)	13
Iodoacetamide (1.0)	25

[a] The tRNA methylase with 1 μM dithioerythritol present was preincubated with a sulfhydryl reagent in potassium phosphate buffer (pH 7.5) for 30 min at 37°C, followed by a 40-min assay at the same temperature. Assay conditions are described in the text.

[b] HO-HgBzO, *p*-Hydroxymercuribenzoate.

iable substrate was used (20 μM SAM and 60 A_{260} units of tRNA per ml), but these values still will give high K_m values for the variable substrate. Both K_m values are in general agreement with those obtained with other tRNA methylases (8–10), although the K_m values for the tRNA(adenine-1)-methylase purified by Glick and Leboy are about 10-fold lower (7).

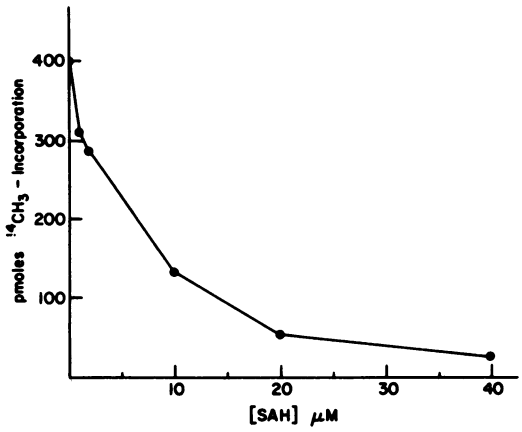

FIG. 8. *Inhibition of supK tRNA methylase by S-adenosylhomocysteine (SAH). The assay as described in the text for the fraction V enzyme was performed with the addition of S-adenosylhomocysteine (Sigma) to the final concentration indicated.*

DISCUSSION

In our earlier work on the *supK* tRNA methylase (21), we found that we could not get quantitative in vitro methylation. The crude assay system we were using was apparently specific for methylation of only one nucleotide, since the tRNA substrate we used was deficient in only one type of methyl group (18). However, due to the presence of one or more nuclease activities, the tRNA substrate in the reaction mixture was digested during the assay, and we often saw a loss of trichloroacetic acid-precipitable radioactivity at late assay times. To quantitate the methyl groups added to tRNA and to identify the tRNA species that accepted methyl groups and the methylated nucleotide itself, we found it necessary to purify and characterize the *supK* tRNA methylase.

The *supK* methylase has properties similar to those of other tRNA methylases. As with other bacterial tRNA methylases, it is somewhat unstable, especially in crude extracts (19). It is inhibited by sulfhydryl reagents and by S-adenosylhomocysteine (7-9). It has kinetic properties like those of most other methylases, with a K_m value of about 2 μM for SAM and 0.3 μM for its substrate tRNA (1, 8, 9), although the K_m values reported for the 1-adenine methylase from rat liver are about 10-fold lower (7). Several tRNA methylases have been purified to near homogeneity (1, 7, 10, 24), and molecular weights have been reported for three of these enzymes. Two forms of the *E. coli* 7-guanine methylase have molecular weights of 1×10^5 and 3×10^5 (1), the N^2-guanine methylase from chicken embryos has a molecular weight of 77,000 (10), and the 1-adenine methylase from rat liver has a molecular weight of 95,000 (7). The *supK* methylase has a molecular weight of 52,000 ± 3,000 and appears to be a single polypeptide chain. It is unstable when homogeneous and might be stabilized by another protein which is removed in the final purification step.

Because the enzyme used for these determinations is about 50% pure (fraction V enzyme), its molecular activity can be estimated by using the maximum velocity obtained from the kinetic studies. A V_{max} of 3.3 pmol of methyl group accepted by tRNA in 1 min was obtained with 0.15 μg of protein. This results in a V_{max} of 22 μmol of methyl group incorporated per min per g of methylase. Using a molecular weight of 52,000 for the methylase that is 50% pure, we determined that the molecular activity is 2.2 min^{-1}, which is low in comparison with that of other enzymes (23). Molecular activities have not been reported for other tRNA methylases; however, they have been reported for one other group of enzymes that have tRNA as a substrate, the aminoacyl-tRNA synthetases (3, 5, 6, 11, 12, 14). Molecular activities for these enzymes range from 8 min^{-1} for phenylalanyl-tRNA synthetase (6) to 1,200 min^{-1} for tryptophanyl-tRNA synthetase (11). The amount of tRNA methylase per cell can also be determined by the method of Calender and Berg (3). The crude extract (Table 1) yielded 4×10^4 U of enzyme from 60 g (wet weight) of cells, or 670 U of enzyme from 10^{12} cells (1 g [wet weight]). From the molecular activity of 2.2, this gives 180 molecules of tRNA methylase per cell. Again this value is lower than values obtained for aminoacyl-tRNA synthetases, which are estimated at between 500 and 6,000 molecules per cell (3, 6, 11). However, when one takes into account the functions of these enzymes, these values would appear to be in the correct order of magnitude. Each aminoacyl-tRNA synthetase must esterify about 5% of the total tRNA of the cell, and it must act many times per tRNA per cell generation since the aminoacyl-tRNA functions repeatedly in protein synthesis. The tRNA methylase, on the other hand, although it has as its substrate about 10% of the total tRNA, must act only once per tRNA molecule per cell generation. Thus, its lower molecular activity and abundance when compared with those of the aminoacyl-tRNA synthetases seem reasonable in the light of its biological function.

The most interesting aspect of this enzyme is that from genetic evidence it appears to be a critical enzyme for *S. typhimurium* (20). This implies that its methylated nucleotide product

in tRNA is absolutely required for cell growth. We have recently acquired several new temperature-sensitive and temperature-lethal *supK* mutants of *S. typhimurium* and are now testing these for temperature-sensitive tRNA methylase activity.

We cannot name this tRNA methylase since we have not yet identified the nucleotide product of the enzyme. We have characterized the product as an unstable methylated pyrimidine derivative (18). It does not have the properties of any previously characterized methylated nucleotide from bacteria. We believe it to be a methyl ester of an already modified pyrimidine nucleotide. One possibility is that it is the methyl ester of V, uridin-5-oxyacetic acid (16, 17). The methylated nucleotide, like V, is found in alanine and serine tRNA's (18). It, however, is apparently not present in valine-tRNA of *S. typhimurium*, which in *E. coli* does contain V.

We are presently attempting to identify this interesting methylated product. We will attempt to elucidate its role in the proper reading of the genetic code and how the deficiency of the methyl group leads to UGA suppression.

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LITERATURE CITED

1. **Aschhoff, H. J., H. Elton, H. H. Arnold, G. Mahal, W. Kersten, and H. Kersten.** 1976. 7-Methylguanine specific tRNA-methyltransferase from *Escherichia coli*. Nucleic Acids Res. **3**:3109–3122.
2. **Atkins, J. F., and S. Ryce.** 1974. UGA and non-triplet suppressor reading of the genetic code. Nature (London) **249**:527–530.
3. **Calender, R., and P. Berg.** 1966. Purification and physical characterization of tyrosyl ribonucleic acid synthetases from *Escherichia coli* and *Bacillus subtilis*. Biochemistry **5**:1681–1690.
4. **Davis, B.** 1963. Disc electrophoresis. II. Methods and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
5. **DeLorenzo, F., and B. N. Ames.** 1970. Histidine regulation in *Salmonella typhimurium*. VII. Purification and general properties of the histidyl transfer ribonucleic acid synthetase. J. Biol. Chem. **245**:1710–1716.
6. **Fangman, W. L., G. Nass, and F. C. Neidhardt.** 1965. Immunological and chemical studies of phenylalanyl sRNA synthetase from *Escherichia coli*. J. Mol. Biol. **13**:202–219.
7. **Glick, J. M., and P. S. Leboy.** 1977. Purification and properties of tRNA(adenine-1)-methyltransferase from rat liver. J. Biol. Chem. **252**:4790–4795.
8. **Glick, J. M., S. Ross, and P. S. Leboy.** 1975. S-adenosyl homocysteine inhibition of three purified tRNA methyltransferases from rat liver. Nucleic Acids Res. **2**:1639–1651.
9. **Hurwitz, J., M. Gold, and M. Anders.** 1964. The enzymatic methylation of ribonucleic acid and deoxyribonucleic acid. IV. The properties of the soluble ribonucleic acid-methylating enzymes. J. Biol. Chem. **239**:3474–3482.
10. **Izzo, P., and R. Gantt.** 1977. Partial purification and characterization of an N^2-guanine RNA methyltransferase from chicken embryos. Biochemistry **16**:3576–3581.
11. **Joseph, D. R., and K. H. Muench.** 1971. Tryptophanyl transfer ribonucleic acid synthetase of *Escherichia coli*. J. Biol. Chem. **246**:7602–7609.
12. **Lapointe, J., and D. Soll.** 1972. Glutamyl transfer ribonucleic acid synthetase of *Escherichia coli*. III. Influencé of the 46K protein on the affinity of the 56K glutamyl transfer ribonucleic acid synthetase for its substrates. J. Biol. Chem. **247**:4982–4985.
13. **Lineweaver, H., and D. Burk.** 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**:658–666.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Makman, M. H., and G. L. Cantoni.** 1965. Isolation of seryl and phenylalanyl ribonucleic acid synthetases from baker's yeast. Biochemistry **4**:1434–1442.
16. **Murao, K., M. Saneyoshi, F. Harada, and S. Nishimura.** 1970. Uridin-5-oxy acetic acid: a new minor constituent from *E. coli* valine transfer RNA I. Biochem. Biophys. Res. Commun. **38**:657–666.
17. **Nishimura, S.** 1972. Minor components in transfer RNA: their characterization, location, and function. Prog. Nucl. Acid Res. Mol. Biol. **12**:50–86.
18. **Pope, W. T., A. Brown, and R. H. Reeves.** 1978. The identification of the tRNA substrates for the *supK* tRNA methylase. Nucleic Acids Res. **5**:1041–1057.
19. **Reeves, R. H., C. R. Cantor, and R. W. Chambers.** 1970. Effect of magnesium ion on the conformation of two highly purified yeast alanine transfer ribonucleic acids. Biochemistry **9**:3993–4001.
20. **Reeves, R. H., and J. R. Roth.** 1971. A recessive UGA suppressor. J. Mol. Biol. **56**:523–533.
21. **Reeves, R. H., and J. R. Roth.** 1975. Transfer ribonucleic acid deficiency found in UGA suppressor strains. J. Bacteriol. **124**:332–340.
22. **Riddle, D. L., and J. R. Roth.** 1972. Frameshift suppressors. III. Effects of suppressor mutations on transfer RNA. J. Mol. Biol. **66**:495–506.
23. **Segel, I. H.** 1975. Enzyme kinetics, behavior and analysis of rapid equilibrium and enzyme systems, p. 79–80. John Wiley and Sons, New York.
24. **Taya, Y., and S. Nishimura.** 1973. Biosynthesis of 5-methylaminomethyl-2-thiouridylate. 1. Isolation of a new tRNA methylase specific for 5-methylaminomethyl-2-thiouridylate. Biochem. Biophys. Res. Commun. **51**:1062–1068.
25. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
26. **Whitiker, J. R.** 1963. Determination of molecular weights of proteins by gel filtration on Sephadex. Anal. Chem. **35**:1950–1953.
27. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.