(130 transfers in the system *sec*-butyl alcohol–1% dichloroacetic acid, 1:1, K = 0.31). The contents of tubes 28–34 were evaporated and washed with ether: yield 99 mg, $[\alpha]_D^{18}$ −20° (*c* 1, 80% HOAc). Amino acid ratios were: Asp, 1.06; Glu, 2.02; Pro, 2.06; Gly, 1.01; Ala, 6.07; Val, 3.93; Leu, 0.99; Tyr, 1.90; NH_3, 2.9; Lys, 1.99; His, 1.97.

The product moved as a single spot on paper electrophoresis at pH 5.3 (0.1 M pyridine acetate) with R_{His} = 0.58, and at pH 1.9 (formic acid–acetic acid–water, 15:10:75, v/v) with R_{His} = 0.53.

References

Braunitzer, G., Gehring-Muller, R., Hilschmann, N., Hilse, K., Hombom, G., Rudloff, V., and Wittmann-Liebold, B. (1961), *Z. Physiol. Chem. 325*, 283.

Gutte, B., and Merrifield, R. B. (1969). *J. Am. Chem. Soc. 91*, 501.

Hofmann, K., Haas, W., Smithers, M. J., and Zanetti, G. (1965), *J. Am. Chem. Soc. 87*, 631.

Loffet, A. (1967), *Experientia 23*, 406.

Manning, M. (1968), *J. Am. Chem. Soc. 90*, 1348.

Merrifield, R. B. (1963), *J. Am. Chem. Soc. 85*, 2149.

Merrifield, R. B. (1969), *Advan. Enzymol. 32*, 221.

Ontjes, D. A., and Anfinsen, C. B. (1969), *Proc. Natl. Acad. Sci. U. S.* (in press).

Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964), *Advan. Protein. Chem. 19*, 74.

Schröder, E., and Gibian, H. (1962), *Ann. 656*, 19.

Shaltiel, S. (1967), *Biochem. Biophys. Res. Commun. 29*, 178.

Stewart. J. M., Mizoguchi, T., and Woolley, D. W. (1969), *J. Med. Chem.* (in press).

Stewart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., Freeman.

Takashima, H., du Vigneaud, V., and Merrifield, R. B. (1968), *J. Am. Chem. Soc. 90*, 1323.

Weygand, F., Steglich, W., and Pietta, P. G. (1967), *Chem. Ber. 100*, 3841.

Studies on Lysyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli**

Robert Stern and Alan Peterkofsky†

ABSTRACT: Purification of lysine transfer ribonucleic acid synthetase from *Escherichia coli* grown in ^{32}P-labeled media revealed less than 1 mole of phosphate/mole of enzyme. This ruled out the possibility of oligonucleotides in the recognition mechanism of this enzyme for its cognate transfer ribonucleic acid. No other cofactors were detected in the purified enzyme preparation.

Attempts to preferentially inhibit one of the two activities, the adenosine triphosphate–pyrophosphate exchange and amino acid transfer activity of the apparently homogeneous protein, using inhibitors and limited proteolytic digestion were unsuccessful. Partial protection of both activities in the presence of some substrates was observed. Protection of activities against Nagarse (substilisin) digestion was particularly marked in the presence of transfer ribonucleic acid and lysine. Other properties of the enzyme are presented including amino acid composition, spectral studies, and examination of the inhibition of activity by sulfhydryl reagents.

Aminoacyl-tRNA formation is the first step in protein synthesis. The enzymes which catalyze this reaction, the aminoacyl-tRNA synthetases, must distinguish their specific tRNAs among a large number of very similar RNA molecules. This recognition by an aminoacyl-tRNA synthetase of its specific tRNA is an ideal model for the study of the mechanism of specificity interaction between proteins and nucleic acids. Does the specificity lie entirely in the protein, or are there other groups which participate in the reaction? Coenzymes or metal ions could be invoked, as well as oligonucleotides which would react with the specific tRNA in a manner parallel to the condon–anticodon interaction. No coenzymes or oligonucleotides have yet been determined in aminoacyl-tRNA synthetase reactions. However, it has rarely been possible to obtain sufficient quantities of purified enzymes to assay for these possible enzymatic factors.

The purification and properties of lysyl-tRNA synthetase from kilogram quantities of *Escherichia coli* cell paste have been reported (Stern and Mehler, 1965b; Stern *et al.*, 1966). The purpose of this communication is to demonstrate the absence of stochiometric levels of nucleotides in *E. coli* lysyl-tRNA synthetase, thus ruling out the possibility of oligonucleotides participating in the recognition mechanism between this enzyme and its tRNA.

This investigation also presents additional properties of the

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enzyme including observations on the absorption spectrum, amino acid composition, molecular weight, pH optimum, and on the stability of the two reactions of the apparently homogeneous enzyme. Lysyl-tRNA synthetase has the property of being the only aminoacyl-tRNA synthetase of *E. coli* which is completely resistant to sulfhydryl-blocking agents (Stern *et al.*, 1966). The present communication presents further observations on the effect of sulfhydryl and sulfhydryl-blocking reagents on the activity of the enzyme.

Materials

Inorganic [^{32}P]pyrophosphate and L-[^{14}C]lysine were purchased from New England Nuclear Corp. All unlabeled amino acids were from the California Corp. for Biochemical Research except L-lysine, which was obtained from Mann Research Laboratories. Unlabeled ATP, as the crystalline sodium salt, and glutathione were obtained from Sigma Chemical Co. Millipore filters (HA, 25 mm, 0.45 μ) were obtained from Millipore Filter Corp, Bedford, Mass.

Pancreatic ribonuclease, bacterial alkaline phosphatase, soybean trypsin inhibitor, α-chymotrypsin, and aldolase were purchased from Worthington Biochemical Corp. *E. coli* strain B tRNA was the product of General Biochemicals.

Nagarse, a subtilisin-like enzyme preparation, was obtained from Teikoku Chemical Industry Co., Ltd., Osaka, Japan, and porcine trypsin from Armour Pharmaceutical Co.

Silica gel G for thin-layer chromatography was the product of Brinkmann Co. DTT[1] (Cleland's Reagent) and 2-ME were purchased from the California Corp. for Biochemical Research and Eastman Organic Chemicals, respectively.

Methods

Lysyl-tRNA synthetase activity was estimated in two ways, by its ability to catalyze an amino acid dependent [^{32}P]ATP pyrophosphate exchange and to catalyze the incorporation of ^{14}C-labeled amino acid into acid-precipitable material, as described previously (Stern and Mehler, 1965b).

Protein was determined spectrophotometrically from the absorption of light at 280 and 260 mμ (Warburg and Christian, 1941).

Sucrose gradient centrifugation was similar to that described by Martin and Ames (1961). The centrifugation was for 18 hr set at 0° at 38,000 rpm in a swinging-bucket rotor (SW-39) designed to fit the Model L Spinco centrifuge (Beckman Instruments, Inc.). At this setting the temperature at the end of the run was 5°. The tubes contained aliquots of enzyme from pooled active peak fraction from DEAE-cellulose chromatography plus adolase and methemoglobin (kindly provided by Dr. R. Resnick). Aldolase activity was determined spectrophotometrically by the method of Racker (1947). Methemoglobin was also determined spectrophotometrically by measuring the optical density at 410 mμ. At the end of the run the gradient tubes were pierced and fractions were collected. s values were calculated by the method of Martin and Ames (1961). A value of $s_{20,w}$ of 7.9 S for aldolase (Stellwagen and Schachman, 1962) and 4.55 S for methemoglobin were used (Benesch *et al.*, 1962).

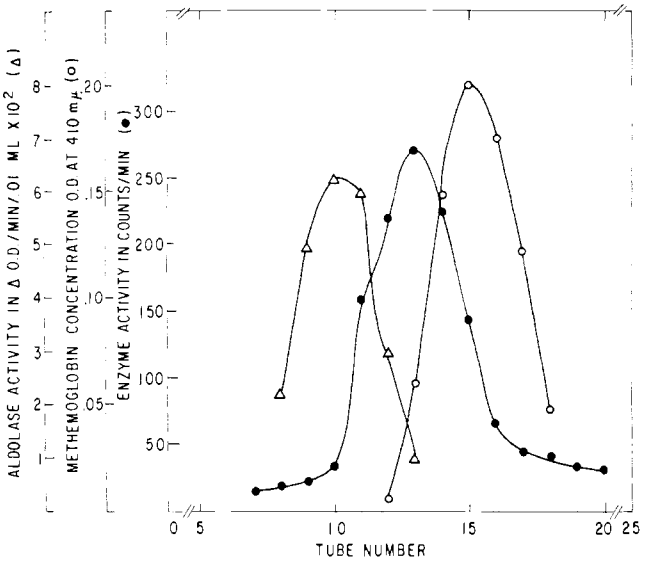

FIGURE 1: Sucrose gradient sedimentation of purified Lys-tRNA synthetase. Aldolase, methemoglobin, and the synthetase in 0.1 ml were layered onto a 4.5-ml gradient (5–20% sucrose) containing 0.05 M Tris buffer (pH 7.5). After 18 hr at 4° at 38,000 rpm in a Spinco Model L preparative ultracentrifuge, 11-drop fractions were collected and assayed for ATP–pyrophosphate exchange (●—●), aldolase activity (Δ—Δ), and methemoglobin (O—O).

Amino Acid Analysis. Protein (1 mg) was dialyzed overnight against distilled H_2O, and hydrolyzed in twice-distilled 5.7 N HCl at 105° for 24 hr in a sealed evacuated tube. The resultant hydrolysate was analyzed in an automatic amino acid analyzer using a single column with a continuous gradient (Piez and Morris, 1960).

End-Group Determination. The fluorodinitrobenzene method was used for the assay of NH_2-terminal amino acids (Fraenkel-Conrat *et al.*, 1955); 5 mg of protein from an hydroxylapatite column eluate was concentrated and incubated with 1-fluoro-2,4-dinitrobenzene for 3 hr at room temperature. A sample of 2.4 mg of pancreatic ribonuclease B was reacted simultaneously in a separate tube.

After dinitrophenylation, the excess DNP was removed with alcohol. The derivatives were then hydrolyzed in 5.7 N HCl in sealed tubes for 12 hr at 100°. The hydrolysates were diluted with water to a concentration of 1 N HCl, treated with ether, evaporated to dryness, dissolved in acetone, and an aliquot was placed on a plate of silica gel G for thin-layer chromatography using the solvent system of Fraenkel-Conrat *et al.* (1955).

Results

The enzyme preparation used throughout these studies was the hydroxylapatite recovered eluate (Stern and Mehler, 1965b). It was free of protein contaminants as judged by the following criteria: constant specific activity across the single optical density peak following chromatography on DEAE-Sephadex, at two different pH values, and on a column of IRC 50, XE 64, and on preparative electrophoresis, and the failure to observe contaminating spots following N-terminal amino acid analysis using a high level of enzyme protein.

[1] Abbreviations used in this work are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PMB, *p*-hydroxymercuribenzoate; 2-ME, 2-mercaptoethanol; IAA, iodoacetamide; DTT, dithiothreitol.

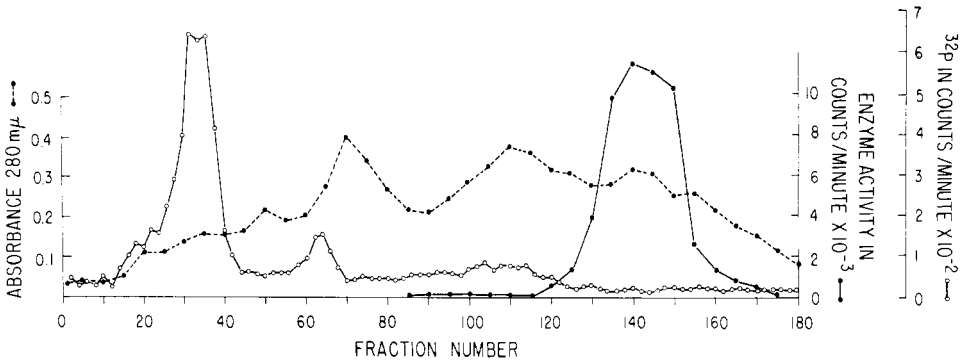

FIGURE 2: The DEAE-cellulose chromatography step in the purification of lysyl-tRNA synthetase from ^{32}P-labeled *E. coli*. Cells were grown in 20 mCi of ^{32}P added to 3 l. of salts medium containing 3 mM phosphate. Cells were harvested and added to 1 kg of unlabeled cell paste. Purification was carried out as described (Stern and Mehler, 1965b). The calcium phosphate eluate described in the purification was applied directly to the DEAE-cellulose column (3.3 × 42 cm) previously equilibrated with 0.05 M potassium phosphate buffer (pH 7.5). The column was washed with 0.1 M potassium phosphate (pH 7.0) buffer until the absorbance at 280 mμ of the effluent fell below 0.02. The column was eluted with a linear gradient formed between 2.0 l. of 0.10 potassium phosphate buffer (pH 7.0, and 2.0 l. of 0.4 M potassium phosphate buffer (pH 6.5), 200 fractions of 20 ml each were collected. The active fractions shown in the figure (130–155) were combined and dialyzed against three changes of 0.02 M potassium phosphate buffer (pH 7.0). The DEAE-cellulose eluate was then applied to an hydroxylapatite column. Absorbance of fractions was measured at 280 mμ (●- - -●). ATP–pyrophosphate exchange activity was measured as described in the legend of Table IV (●—●). The ^{32}P content of fractions was measured by precipitating 0.25-ml aliquot with 10% trichloroacetic acid at 0°, washing onto a Millipore filter, drying, and counting in a Nuclear-Chicago thin-window gas-flow counter (O—O).

Sucrose Gradient Sedimentations. Zone sedimentation velocity in a sucrose gradient using methemoglobin and aldolase as markers gave an $s_{20,w}$ of 5.6 S (Figure 1). This value was consistent with the sedimentation behavior of a protein with a molecular weight of 100,000 (Edsall, 1953). This agrees with published data for this enzyme (Waldenström, 1968).

TABLE I: Amino Acid Composition of Lysyl-tRNA Synthetase from *E. coli*.[a]

Amino Acid	Residues/1000
Aspartic acid	107.9
Threonine	51.5
Serine	47.1
Glutamic acid	134.2
Proline	0.5
Glycine	95.4
Alanine	115.6
Half-cystine[b]	6.5
Valine	82.7
Methionine[c]	4.0
Isoleucine	60.1
Leucine	94.4
Tyrosine	12.3
Phenylalanine	39.3
NH_3^+	139.1
Lysine	53.8
Histidine	22.3
Arginine	59.9

[a] These values represent analysis of an aliquot of enzyme taken from hydroxylapatite fractionation. The protein was fully active before being hydrolyzed. Amino acid analysis was carried out as indicated under Methods. [b] Analyzed as cysteic acid. [c] Analyzed as the methionine sulfoxides.

Amino Acid Composition. The amino acid composition of the purified enzyme is given in Table I. The values compared with those for "stable" amino acids of *E. coli* determined by Sueoka (1961) indicate that the amino acid composition is similar to that of total protein of *E. coli*, except for the low content of proline and cysteine. The 6.5 half-cystine residues present as cysteic acid per 1000 residues represents 5 residues per 100,000 molecular weight protein of 800 residues. Two SH groups per mole can be titrated with DTNB in the native state, and a third is observed in the presence of urea (Stern *et al.*, 1966). Therefore, two additional half-cystines are present in the molecule. These may be present as a single disulfide bridge. However, this cannot be concluded from the present data alone.

N-Terminal Amino Acid. No amino acid spot was detected following chromatography of the hydrolyzed dinitrophenylated protein. L-Lysine was obtained from the sample of ribonuclease which was processed simultaneously with the lysyl-tRNA synthetase (Anfinsen *et al.*, 1954). In addition, ten times the level of enzyme was chromatographed in an attempt to detect other amino acid derivatives from any possible protein contaminants in the enzyme preparation. No contaminating spot was observed.

Optical Studies. The absorbancy profile of the enzyme preparation between wavelengths of 240 and 330 mμ was compatible with the pattern of a pure protein. The ratio of absorbance at 280 and 260 mμ was 1.8. However the possibility that small oligonucleotides were bound to the enzyme could not be ruled out by the spectrum alone.

In an attempt to look for nucleotides or enzyme cofactors, a hot perchloric acid extract of the protein was prepared; 3 mg/ml of protein was incubated in 7% perchloric acid for 10 min at 100°. The extract had little absorbance between 250 and 300 mμ and appeared identical with the spectrum of the hot extract of the same amount of crystalline bovine serum albumin.

A yellow color was consistently observed in concentrated (10–12 mg/ml) preparations of the pure enzyme. The absorp-

TABLE II: [^{32}P]Enzyme Preparation.[a]

Purification Step	Sp Act. (cpm/mg)	Total Act. (cpm)
Extract	1.9×10^4	2×10^9
Streptomycin supernatant	2.0×10^4	4×10^8
$Ca_3(PO_4)_2$	1.0×10^4	8×10^6
DEAE eluate	1.2×10^2	2×10^4
Hydroxylapatite eluate	1.0×10^2	6×10^3

[a] Purification of lysyl-tRNA synthetase from ^{32}P-labeled *Escherichia coli*. Cells were grown in 20 mCi of ^{32}P added to 3 l. of salts medium containing 3 mM phosphate. Harvested cells were added to 1 kg of unlabeled cell paste and a normal purification was carried out (Stern and Mehler, 1965b). Cells grown in 3 l. of medium containing 20 mCi of ^{32}P and 9 mmoles of phosphate was added to the kilogram of cell paste representing an approximate dilution of 1 to 150 by weight. Allowing a single half-life for the period of the experiment and 40% efficiency for the Nuclear-Chicago gas-flow counter, 1.5×10^4 cpm was estimated for each μmole of phosphate; 6×10^3 cpm and 60 mg of protein following the last step of the purification procedure was compatible with 0.7 mole of phosphate/mole of enzyme of 100,000 molecular weight.

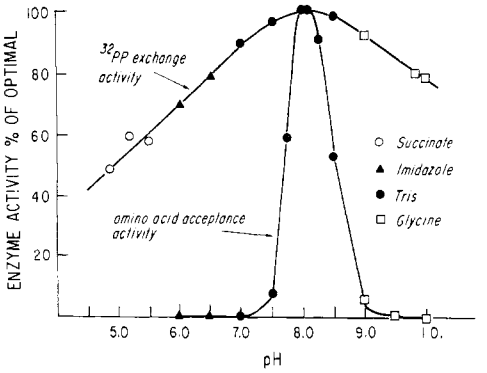

FIGURE 3: pH optimum curve of the two reactions of lysyl-tRNA synthetase, the ATP-pyrophosphate exchange and the tRNA amino acid acceptance activity. Activities were measured as described in the legend of Table IV, except that the buffers used were indicated in the figure.

tion at 310 mμ of the preparation was 1 optical density unit. Absorption decreased gradually with increasing wavelength to near base line at 600 mμ. The optical density profile of the protein solution (11.1 mg/ml) was documented in the visible range. At 320 mμ, 0.8 optical density unit was observed; at 400 mμ, 0.4 optical density unit; at 450 mμ, 0.28 optical density unit; and at 650 mμ, 0.16 optical density unit; and the optical density continued to trail into the blue region. No characteristic peak could be observed between 300 and 600 mμ. The yellow color could not be removed by ammonium sulfate precipitation and resuspension of the enzyme, nor by the addition of Norit. No color was observed in the hot perchloric acid extract of the protein.

^{32}P-Labeling Studies. To examine the question of possible nucleotide cofactors or contamination more closely, the enzyme was purified from *E. coli* that had been grown in ^{32}P-labeled minimal salts low phosphate medium. [^{32}P]Phosphate (20 mCi) was added to the medium, inoculated with *E. coli* B, and grown overnight. Cells were harvested by centrifugation and added to 1 kg of cell paste and the usual purification procedure was carried out. The results of this purification are presented in Table II and Figure 2. The final product contained radioactivity compatible with less than 1 mole of phosphate/mole of enzyme, assuming a molecular weight of 100,000. The residual phosphate could not be removed with incubation for 30 min at 37° with bacterial alkaline phosphatase.

Attempts to Crystallize the Protein. Attempts to crystallize the enzyme in the absence and presence of various combinations of Mg, ATP, and lysine, were unsuccessful. Enzyme activity precipitated between 50 and 60% ammonium sulfate at 0° in the crude state. However precipitation occurred at a much lower concentration of ammonium sulfate with a purified, concentrated preparation of enzyme. With 12 mg/ml of protein in 0.01 M Tris (pH 8.0), turbidity occurred at 30% ammonium sulfate at 0°. There was a time-dependent loss of activity in ammonium sulfate, 30% of the activity being lost in 6 hr which occurred with both crude and purified preparations of the enzyme.

pH Optimum and Inhibition Studies. Optimum pH of both the ATP–pyrophosphate exchange activity and the transfer reaction was pH 8.0 (Figure 3). However there was a marked difference in the shape of the pH curve of the two reactions. The ATP–pyrophosphate exchange activity showed only a slight pH dependency; 90% of the activity remained at pH values 7.0 or 9.0 and 70% remained at pH 6.0. On the other hand, only 10% of the transfer reaction was detected at pH 7.5. A time course of the transfer reaction at pH 7.5 and at pH 8.0 is illustrated in Figure 4.

Exchange activity was inhibited 60% in the presence of phosphate buffer. Inhibition was also observed in the presence of cacodylate buffer. In addition, an apparent shift in pH optimum occurred to pH 6.0. This phenomenon is also observed with valyl-tRNA synthetase (Bergmann *et al.*, 1961).

A series of experiments were undertaken in an attempt to separate the two activities of the single protein. High salt concentrations had no effect on the two reactions of the enzyme (Table III). Both the ATP–pyrophosphate exchange and the tRNA acylation reaction were diminished in the presence of sulfhydryl compounds such as 2-ME and DTT (Table III and Figure 5a,b). The apparent activity decreased as the concentration of the sulfhydryl reagents increased, 90% of the activity was destroyed in the presence of 0.1 M DTT or 1.0 M 2-ME. This loss of activity could not be recovered by dilution or by dialysis of the enzyme using sulfhydryl free buffers. The effect of a particular concentration of sulfhydryl reagent was not increased with prolonged incubation with the reagent at 37°. Glutathione also caused a decrease in activity of the enzyme (Table III) though the profile of inactivation differed from the other reagents (Figure 5c).

Urea at 2 M was without effect on the two reactions while 6 M urea caused complete inactivation. The enzyme activities were only partially restored following removal of urea (Table III). Treatment of the enzyme PMB and DTNB in the absence of and in the presence of 2 and 6 M urea also failed to differentially inhibit one of the two reactions (Stern *et al.*, 1966).

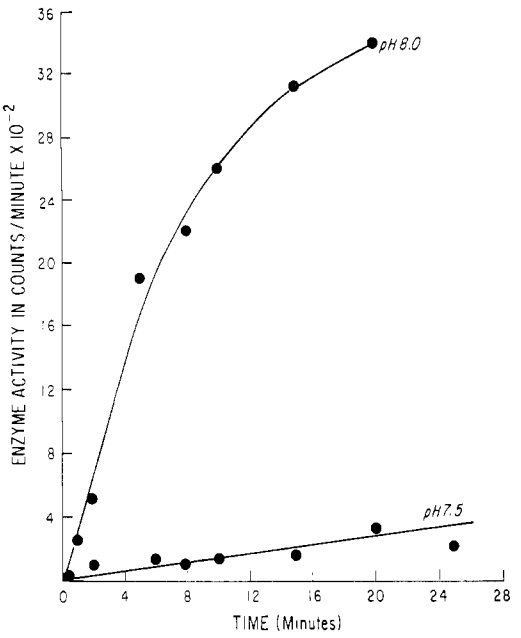

FIGURE 4: A time course of the amino acid acceptance activity of lysyl-tRNA synthetase at pH 7.5 and 8.0. The assay was performed as described in the legend of Table IV. Aliquots of the reaction mixture were removed at the indicated times and the reaction was stopped with the addition of 10% trichloroacetic acid at 0°.

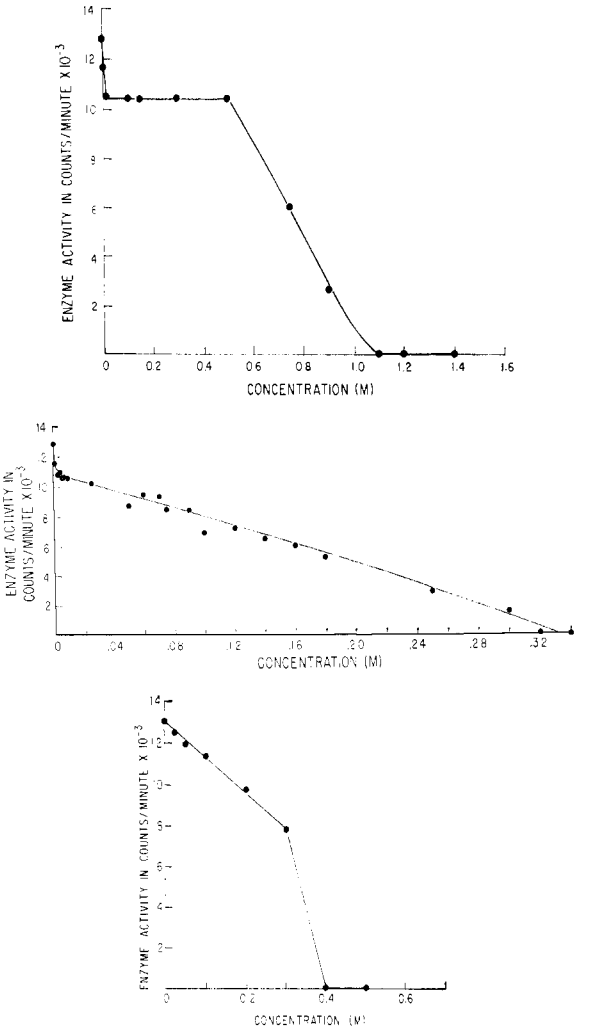

FIGURE 5: Inhibition of ATP–pyrophosphate exchange activity in the presence of 2-mercaptoethanol (a), dithiothreitol (b), and glutathione (c) in the reaction mixture at the concentrations indicated in the figure. Activity was assayed as described in the legend of Table IV.

Sulfhydryl-blocking reagents such as PMB and $HgCl_2$ at concentrations up to 5×10^{-3} M exerted no effect on the ATP–pyrophosphate exchange reaction (Table III). Their effect on the tRNA acylation reaction may have been due to their reaction with tRNA rather than with the enzyme (see Discussion). IAA, NEM, or DTNB (Ellman, 1959) at 10^{-3} M were ineffective in both reactions of the enzyme. The resistance of this enzyme to sulfhydryl-blocking reagents is unique among the aminoacyl-RNA synthetase enzymes of *E. coli* and has been examined in a previous publication (Stern *et al.*, 1966).

The presence of tRNA in the ATP–pyrophosphate exchange reaction mixture had no effect on the rate of the ATP–pyrophosphate reaction. Amino acids on the same metabolic pathway as lysine (such as methionine, threonine, aspartic acid) and other basic amino acids (such as arginine, glutamine, and asparagine) also failed to influence the reaction rate.

Limited Proteolytic Digestion. Experiments were undertaken to determine if an enzyme preparation could be obtained that had lost the ability to react with specific tRNA, yet would retain the ability to catalyze an ATP–pyrophosphate exchange. The effects of proteolytic digestion by limiting amounts of trypsin, chymotrypsin, and Nagarse, a subtilisin-like preparation, were examined (Table IV).

Trypsin digestion totally inactivated both the ATP–pyrophosphate reaction and the tRNA acylation reaction when the enzyme was exposed to 2×10^{-2} mg/ml of the protease (Table IV, expt 1). Chymotrypsin inactivated the exchange reaction at a concentration of 5×10^{-4} mg/ml while 55% of the acylation reaction remained (Table IV, expt 2). Nagarse, a subtilisin-like preparation was effective in inactivating both reactions when added at 2×10^{-3} mg/ml (Table IV, expt 3). In no case was it possible to achieve a complete dissociation of the exchange activity from the tRNA acylation reaction.

Various substrates were added to the reaction mixtures in an attempt to obtain preferential protection of the enzyme from proteolytic degradation. The presence of lysine in the incubation mixture during the proteolytic digestion with Nagarse was particularly effective in protecting the enzyme from degradation. AMP in the reaction mixture reduced the protective effect of lysine but had no effect when incubated alone with the enzyme. tRNA was partially effective in protecting activity. ATP, ATP, and Mg^{2+}, and pyrophosphate were without effect (Table IV, expt 4). However in none of these cases was it possible to effect a separation of the two activities.

The tRNA acylation reaction as measured in these experiments is the sum of the ATP–pyrophosphate exchange reaction and the tRNA acylation. The second reaction is much slower than the first in all aminoacyl-tRNA synthetases which have been studied. In the case of the lysine enzyme from *E. coli*, 1 mg of the purified protein supports the exchange of 312 μ-moles of [^{32}P]pyrophosphate into ATP and 500 mμmoles of L-lysine into tRNA under standard reaction mixture conditions

TABLE III: Effect of Additions to Preincubation Mixture and Assay Mixtures on the Two Activities of Lysyl-tRNA Synthetase.

Addition	Concn during Preincubn (20 min at 37°) and in the Reaction Mixture, Except Where Indicated	% Act. Remaining	
		ATP–Pyrophosphate Exchange Assay	tRNA Aminoacylation Assay
Salt effect			
NH_4Cl	1.0×10^{-1}	100	100
KCl	3.5×10^{-1}	100	100
Sulfhydryl reagents			
DTT	5.0×10^{-2}	20	14
DTT 4-hr preincubation	5.0×10^{-2}	22	10
DTT	1.0×10^{-1}	0	0
2-ME	0.5	55	20
2-ME	1.0	0	0
2-ME	1.0		
2-ME followed by dialysis		8	0
GSH	5×10^{-3}	96	100
Urea			
Urea	2.0	100	100
Urea	6.0	0	0
Urea followed by dialysis	6.0	70	50
Sulfhydryl-blocking reagents			
DTNB	1.0×10^{-3}	100	100
NEM	1.0×10^{-3}	100	100
IAA	1.5×10^{-4}	100	100
PMB[a]	1.2×10^{-3}	100	7
$HgCl_2$	1.5×10^{-3}	98	12
PMB[b] and 2-ME	1.2×10^{-3}		
	5.0×10^{-3}	90	86

[a] Preincubation with lysine, ATP, and Mg^{2+} did not alter the resistance of the enzyme to PMB. [b] PMB was incubated with the enzyme for 20 min at 37° followed by the addition of 2-ME for an additional 10 min.

(Stern and Mehler, 1965b). The paradox of apparent complete inactivation of the exchange reaction in the presence of residual tRNA acylation activity may be explained by the difference in rate and difference in limits of detection between the two activities.

Discussion

The molecular weights for the aminoacyl-tRNA synthetases which have been examined to date are all near integers of 50,000. The molecular weights for *E. coli* aminoacyl-tRNA synthetases are Ile, 112,000 (Norris and Berg, 1964); Glu, 50,000 (Lazzarini and Mehler, 1964); and Tyr, 98,000 (Calendar and Berg, 1965). From yeast, values for Ser-RNA synthetase of 89,000 and for Phe-RNA synthetase of 180,000 have been reported (Makman and Cantoni, 1965) and for lysine and valine, 112,000 and 116,000, respectively (Lagerkvist and Waldenström, 1965). The molecular weight of the lysyl-tRNA synthetase determined in the present experiments was 100,000. The data above suggest that all aminoacyl synthetase proteins might be monomers, dimers, or tetramers of a 50,000 molecular weight subunit. If this is the case, then the ^{32}P data suggest that only one if any of the two subunits can be phosphorylated.

Aminoacyl-tRNA synthetase preparations of high purity from several sources have had varying amounts of nucleotides associated with the enzyme. An absorption spectrum compatible with a 3% nucleotide content is observed in the case of the alanine enzyme of liver (Webster, 1961) and the tryptophan enzyme of pancreas (Davie *et al.*, 1956). In the later preparation the main component of nucleotide present was tentatively identified as guanosine monophosphate. Deutscher (1967) has reported that glutamyl-tRNA synthetase from rat liver contains 1.5% nucleotide material. This material is not essential for enzyme activity. Removal, while not affecting activity, does increase lability of the enzyme upon storage.

In the case of the lysine enzyme the ratio of light absorbed at 280 and 260 mμ is 1.80 compatible with less than one nucleotide per enzyme molecule (Warburg and Christian, 1941). From the present results, it is unlikely that an oligonucleotide can be invoked as part of the recognition site between the lysine enzyme and lysyl-tRNA. Less than a single phosphate per mole of enzyme remained after the last step in the purification (Table II). A single molecule of lysine adenylate (Norris

TABLE IV[a]

Trypsin Added (mg)	% Act. Remaining: ATP–Pyrophosphate Exchange Assay	% Act. Remaining: tRNA Acylation Reaction
Expt 1: Trypsin[b]		
10^{-3}	0	0
10^{-4}	40	88
10^{-5}	100	100
Chymotrypsin Added (mg)		
Expt 2: Chymotrypsin[c]		
2.5×10^{-5}	0	55
2.5×10^{-6}	15	75
2.5×10^{-7}	30	100
2.5×10^{-8}	100	100
Nagarse Added (mg)		
Expt 3: Nagarse[d]		
10^{-4}	0	0
10^{-5}	12	50
10^{-6}	40	100
10^{-7}	70	100

Additions	Expt 4: Nagarse Plus Additions[e] % Act. Remaining ATP–Pyrophosphate Exchange Assay: 10^{-4} mg of Nagarse	10^{-5} mg of Nagarse
None	0	12
tRNA (0.5 mg)	18	48
ATP	0	12
ATP and Mg^{2+}	0	12
Pyrophosphate	0	10
AMP	0	18
AMP and L-lysine	18	48
L-Lysine	30	90

[a] Stability of Lys-tRNA synthetase to trypsin digestion (expt 1), chymotrypsin digestion (expt 2), and to Nagarse digestion in the absence and presence of various substrates (expt 3 and 4, respectively). ATP–pyrophosphate exchange assay: The reaction mixtures contained in 1.0 ml: 100 μmoles of Tris buffer (pH 8.0), 8 μmoles of $MgCl_2$, 2 μmoles of ATP, 2 μmoles of $Na_4P_2O_7$, sufficient [^{32}P]$Na_4P_2O_7$ to yield a specific activity of approximately 10^5 cpm/μmole, and 2 μmoles of L-lysine. The reaction was begun by adding 1 μg of enzyme which had been preincubated as indicated. The incubation was carried out at 37° for 15 min. The reaction was stopped with 0.5 ml of 7% perchloric acid which was 0.2 M in $Na_4P_2O_7$, and 1 ml of 7% acid-treated Norit was added. The reaction mixtures were then collected on a 25-mm Millipore filter and washed with three 20-ml portions of distilled water. The filters were glued onto planchets, dried, and counted on a Nuclear-Chicago thin-window gas-flow counter. tRNA amino acylation assay: The reaction mixture contained in 0.2 ml: 25 μmoles of Tris buffer (pH 8.0), 1 μmole of $MgCl_2$, 0.2 μmole of ATP, and 10 mμmoles of L-[^{14}C]lysine, and 0.5 mg of tRNA. The reaction was begun by the addition of 1 μg of enzyme which had been preincubated as indicated and terminated after 6-min incubation at 37° by the addition of 1 ml of 10% trichloroacetic acid. The resulting suspensions were chilled for 10 min and filtered through a Millipore filter and washed three times with 20-ml volumes of 1% trichloroacetic acid. The precipitates together with the Millipore filters were then placed in a counting vial, neutralized with 1.5 N NH_4OH, and dissolved in 10 ml of the liquid scintillator described by Bray (1960). [b] The 30-min preincubation mixture which was with trypsin in 10^{-3} M HCl, 5×10^{-3} M $CaCl_2$, contained 4 μg of pure enzyme preparation in 50 μl. The solution was incubated at room temperature for 10 min then placed on ice and 20 μg of soybean trypsin inhibitor was added. A 10-μl aliquot of this preparation was added to the standard reaction mixtures. [c] Chymotrypsin in 10^{-3} M HCl was added to 4 μg of purified enzyme preparation in a total volume of 50 μl of incubated at 37° for 15 min. Aliquots of 10 μl were removed and immediately added to the reaction mixtures. [d] Nagarse in 10^{-2} M Tris (pH 8.0)–5×10^{-3} M $CaCl_2$ was added to 1 μg of enzyme in a total volume of 30 μl and incubated at room temperature for 10 min. DFP was then added to a final concentration of 2×10^{-2} M and the entire solution was added to the reaction mixture. [e] Conditions are the same as those listed under expt 3. The addition of ATP, Mg^{2+}, lysine, and pyrophosphate to the preincubation were in the amount found in the standard ATP–pyrophosphate reaction mixture. In each case, when the addition was made to the preincubation solution, the reagent was omitted from the subsequent enzyme reaction mixture. AMP was used at the same concentration as ATP.

and Berg, 1964) may be bound to some of the enzyme molecules. However this cannot be concluded from the present data.

The model of an aminoacyl-RNA synthetase from the work of Bergmann *et al.* (1961) and Norris and Berg (1964) suggests that the protein binds aminoacyl adenylate at one site, and that a separate site is involved in amino acid transfer to the specific tRNA. Differences in the specificity of the two reactions have been observed in the case of *E. coli* isoleucyl-tRNA synthetase and *E. coli* valyl-tRNA synthetase (Bergmann *et al.*, 1961) and in the case of *E. coli* lysyl-tRNA synthetase (Stern and Mehler, 1965b). In each of these cases the exchange reaction is less specific than the transfer step. In the present communication experiments were described which attempted

to preferentially inactivate one of the two activities to demonstrate that the two reaction sites are resolvable. High salt concentrations, sulfhydryl-containing reagents, sulfhydryl-blocking reagents, urea, and limiting concentrations of proteolytic enzymes were used (Tables III and IV, Figure 5). In no case was it possible to demonstrate inhibition of the transfer reaction without an effect on the exchange reaction. No change in reactivity of either activity was observed in the presence of high salt.

The addition of monovalent cations produces a variable response in the synthetase reactions in general. The ATP–pyrophosphate exchange activity of alanyl-tRNA synthetase of pig liver is stimulated by K^+ and Rb^+ (Schweet and Allen, 1958) and the leucyl-tRNA synthetase of *Pseudomonas aeruginosa* requires NH_4^+ ions for the transfer reaction (Kaziro *et al.*, 1968). However, no such effect was elicited from the lysine enzyme (Table III). It was observed that phosphate inhibited enzyme activity as did cacocylate ions. Ammonium sulfate caused a progressive instability of the enzyme upon storage though it had no effect on activity directly.

Differential inhibition of the two synthetase reactions is reported in the case of the methionyl enzyme from *E. coli* (Cassio, 1968). This has been cited as evidence for the distinct and resolvable areas on the protein specific for each of the two reactions (Papas and Mehler, 1968). The two reactions of methionyl-tRNA synthetase are inhibited by PMB. The presence of L-methionyl adenylate protects the exchange reaction from PMB inhibition without protecting the transfer reaction. However, there is another possible explanation for the observation. PMB and other mercurials react with the nitrogens and the amino groups of the bases in RNA. The absorption maxima of RNA is shifted from 260 to 276 mμ (Simpson, 1964). This association of mercuric ions with tRNA can cause a loss of transfer activity (Stern *et al.*, 1966). The lysyl tRNA synthetase exchange activity is not inhibited by PMB, while the transfer activity is apparently completely inhibited. However, other sulfhydryl reagents such as NEM and DTNB have no effect on either activity. Thus, it is likely that the PMB effect is due to its reaction with tRNA in both the case of the lysine enzyme and the methionine enzyme. It is unlikely that a differential inhibition on the two activities has been effected in either of the two enzymes.

Lysyl-tRNA synthetases from *E. coli* (Kalousek and Rychlík, 1965) and wheat germ (Moustafa, 1964) are inhibited by PMB but not by other sulfhydryl-blocking reagents such as IAA and NEM. These observations are also explained by the action of PMB on tRNA, rather than on the enzyme. The only resolution of the two reactive sites of an amino acid activating enzyme appears to be the case of prolyl-tRNA synthetase from *E. coli* (Papas and Mehler, 1968). Cold inactivation of the transfer activity of this enzyme occurs after purification, while the exchange activity remains unimpaired.

The inhibition of lysyl-tRNA synthetase by sulfhydryl reagents (Figure 5) fell into two categories. Reagents such as 2-ME and DTT caused an initial rapid decrease in activity, to 85% of the activity of the untreated enzyme, followed by a gradual decline in activity (Figure 5a,b). A linear decrease in ATP–pyrophosphate exchange activity was noted with increasing levels of GSH (Figure 5c).

In one other case has a distinction between the two classes of sulfhydryl reagents been observed. Black and Hazel (1969) have reported that when inorganic sulfide is included in the enzyme assay, activity of glycyl-tRNA synthetase from yeast becomes dependent upon GSH. The enzyme shows no such dependence in the absence of sulfide. GSH cannot be replaced by 2-ME or DTT in this system. These data are interpreted by the authors to indicate that disulfide bonds can be generated in the isolated synthetase which are resistant to ordinary thiol reduction but which can be cleaved by sulfides to form a mixed disulfide, an inactive form of the enzyme. The enzyme can then be reduced by the relatively mild reducing agent GSH to regenerate active enzyme.

In the case of the lysine enzyme, a disulfide bond not absolutely essential for activity may have been reduced by low concentrations of 2-ME and DTT. Increasing concentrations of reagents were then only slowly able to continue inactivating the protein (Figure 5). The break in the curve of 2-ME inactivation cannot be explained, though at this high concentration, an organic solvent affect of 2-ME is plausible.

A difference between the two classes of sulfhydryl reagents was also observed in their effect upon enzyme stability during storage. GSH was inhibitory when added to the reaction mixture, but it increased the stability of the enzyme on storage (Stern and Mehler, 1965b) while 2-ME and DTT caused complete inactivation of the enzyme under these conditions.

The aminoacyl-tRNA synthetases show great variation in their physical and chemical properties including variation in stability, response to inhibitors and in the case of the lysine enzyme, response to sulfhydryl-containing compounds. Among the synthetases of *E. coli*, the lysine enzyme is the only one which is impervious to sulfhydryl-blocking reagents (Stern *et al.*, 1966) and which is actually inhibited by sulfhydryl-containing compounds. No pattern has yet emerged from the mass of data on the properties of aminoacyl-tRNA synthetases. By criterion of physical and chemical properties there is no indication of a genetic or evolutionary relatedness among enzymes among which such relatedness might be expected, among synthetases of amino acids on the same metabolic pathway or synthetases of amino acids with similar codon assignments. However, amino acid sequence data would be needed to establish this point definitively. We can conclude that each synthetase must be treated as a unique entity and as has been demonstrated in the case of the lysine enzyme, results obtained with one enzyme are not applicable to any other.

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References

Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page, J., and Carroll, W. R. (1954), *J. Biol. Chem. 207*, 201.

Benesch, R., Benesch, R. E., Ranney, H. M., and Jacobs, A. S. (1962), *Nature 194*, 840.

Bergmann, F. H., Berg, P., and Dieckmann, M. (1961), *J. Biol. Chem. 236*, 1735.

Black, S., and Hazel, B. (1969), *J. Biol. Chem. 244*, 1956.

Bray, G. (1960), *Anal. Biochem. 1*, 279.

Calendar, R., and Berg, P. (1965), *Fed. Proc. 24*, 217.
Cassio, D. (1968), *European J. Biochem. 4*, 222.
Davie, E. W., Koningsberger, U. V., and Lipmann, F. (1956), *Arch. Biochem. Biophys. 65*, 21.
Edsall, J. T. (1953), *Proteins 1*, 634.
Deutscher, M. (1967), *J. Biol. Chem. 242*, 1123.
Ellman, G. L. (1959), *Arch. Bioch. Biophys. 82*, 70.
Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 359.
Kalousek, F., and Rychlík, I. (1965), *Collection Czech. Chem. Commun. 30*, 3909.
Kaziro, Y., Takahashi, Y., and Inoue, N. (1968), *J. Biol. Chem. 240*, PC 2264.
Lagerkvist, V., and Waldenström, J. (1965), *J. Biol. Chem. 240*, PC 2265.
Lazzarini, R. A., and Mehler, A. H. (1964), *Biochemistry 3*, 1445.
Makman, M. H., and Cantoni, G. L. (1965), *Biochemistry 4*, 1434.
Martin, R., and Ames, B. (1961), *J. Biol. Chem. 236*, 1372.
Moustafa, E. (1964), *Biochim. Biophys. Acta 91*, 421.
Norris A. T., and Berg, P. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 330.
Papas, T. S., Mehler, A. H. (1968), *J. Biol. Chem. 243*, 3767.
Piez, K. A., and Morris, L. (1960), *Anal. Biochem. 1*, 187.
Racker, E. (1947), *J. Biol. Chem. 167*, 843.
Schweet, R. A., and Allen, E. H. (1958), *J. Biol. Chem. 233*, 1104.
Simpson, R. B. (1964), *J. Am. Chem. Soc. 86*, 2059.
Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 1*, 1056.
Stern, R., DeLuca, M., Mehler, A. H., and McElroy, W. D. (1966), *Biochemistry 5*, 126
Stern, R., and Mehler, A. H. (1965a), *Federation Proc. 24*, 217.
Stern, R., and Mehler, A. H. (1965b), *Biochem. Z. 342*, 400.
Sueoka, N. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1141.
Waldenström, J. (1968), *European J. Biochem. 3*, 483.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Webster, G. C. (1961), *Biochim. Biophys. Acta 49*, 141.

Polymers Containing 2′-*O*-Methylnucleotides. II. Synthesis of Heteropolymers*

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ABSTRACT: Heteropolymers containing 2′-*O*-methyladenylic acid, 2′-*O*-methylcytidylic acid, and nonmethylated nucleotides have been synthesized with polynucleotide phosphorylase from their corresponding nucleoside 5′-diphosphates. The distribution of 2′-*O*-methylnucleotides in these polymers was not completely random but occurred in pairs. The frequency of this paired incorporation indicates some form of cooperative polymerization of 2′-*O*-methylnucleotides in the presence of nonmethylated nucleotides. The addition of dimethyl sulfoxide to the polymerization reaction enhanced the random incorporation of 2′-*O*-methyladenosine 5′-diphosphate in the presence of cytidine 5′-diphosphate but had little effect on the incorporation of 2′-*O*-methylcytidine 5′-diphosphate in the presence of uridine 5′-diphosphate. Certain restrictions on the formation of heteropolymers by *Micrococcus lysodeikticus* polynucleotide phosphorylase were noted. 2′-*O*-Methyladenosine 5′-diphosphate formed heteropolymers with cytidine 5′diphosphate and uridine 5′-diphosphate but not guanosine 5′-diphosphate and adenosine 5′-diphosphate. 2′-*O*-Methylcytidine 5′-diphosphate formed heteropolymers with uridine 5′-diphosphate but would not form a homopolymer nor would it form heteropolymers with cytidine 5′-diphosphate, adenosine 5′-diphosphate, or guanosine 5′-diphosphate. A heteropolymer containing 2′-*O*-methyladenosine, 2′-*O*-methylcytidine, and uridine was synthesized from the corresponding nucleoside 5′-diphosphates.

Polynucleotide phosphorylase is known to catalyze the polymerization of 2′-*O*-methyladenosine 5′-diphosphate forming a large molecular weight homopolymer, poly-2′-*O*-methyladenylic acid (Rottman and Heinlein, 1968). Evidence for the formation of a heteropolymer containing 2′-*O*-methyladenylic acid and uridylic acid was also presented. However, the possibility of forming RNA molecules more closely approximating those isolated from natural sources in both 2′-*O*-methyl content and distribution was not examined.

The widespread distribution of 2′-*O*-methylnucleotides in RNA from many organisms has been amply demonstrated (Smith and Dunn, 1959; Biswas and Meyers, 1960; Hall, 1964; Tamaoki and Lane, 1968). Information concerning the possible function of this modification is minimal (Starr and Sells, 1969). Methylation of preribosomal 45S RNA in HeLa cells has been reported to be a prerequisite for its maturation and processing into 18S and 28S RNA (Vaughn *et al.*, 1967). This processing may involve the 2′-*O*-methyl group since the major site of methylation in

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