Verma, I. M., Meuth, N. L., Bromfeld, E., Manly, K. F., and Baltimore, D. (1971), *Nature New Biol. 233*, 131.
Wells, R. D., and Blair, J. E. (1967), *J. Mol. Biol. 27*, 273.
Wells, R. D., Jacob, T. M., Narang, S. A., and Khorana, H. G. (1967), *J. Mol. Biol. 27*, 237.
Wells, R. D., and Larson, J. E. (1970), *J. Mol. Biol. 49*, 319.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., and Cantor, C. R. (1970), *J. Mol. Biol. 54*, 465.

Transfer Ribonucleic Acid Methylases of Nucleoli Isolated from a Rat Tumor*

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ABSTRACT: When nucleoli were isolated from Novikoff ascites tumor cells of the rat and incubated with L-[*methyl*-^{3}H]S-adenosylmethionine in a suitable medium, incorporation of the methyl groups into the ribose of preribosomal RNA was observed. When *Escherichia coli* tRNA was added to the medium, this exogenous tRNA was methylated in addition, but the label was located on the purine and pyrimidine bases. The enzymes responsible for methylation of the bases of heterologous tRNA were extracted with 0.5 M NH_4Cl medium from the nucleoli and compared with pH 5-precipitated tRNA methylases derived mainly from cytoplasmic or soluble portions of the cells. The nucleolar enzymes represented about 5% of the total cellular tRNA methylase activity extractable from the cells. The activities of the nucleolar and "cell-soluble, pH 5" preparations were similar in response to pH, ionic strength, and ATP concentration, and differed somewhat in response to NaF and $MgCl_2$ concentrations. The major products with both preparations were 1-methyladenine, 7-methylguanine, 1-methylguanine, N^2-methylguanine, N^2-dimethylguanine, 5-methylcytosine, thymine, and 5-hydroxymethyluracil. The proportion of the methylated bases differed considerably; for example, with the nucleolar preparation 69% of the label was found in methylcytosine and 15% in methylguanines, while with the cell-soluble, pH 5 enzyme, 13% was in methylcytosine and 60% was in the guanine derivatives. When cells were labeled *in vivo* with [*methyl*-^{3}H]-methionine, the patterns of labeling of endogenous 4–6S RNAs had the same trend; for example, 50% of the nucleolar sRNA methyl groups were in cytosine derivatives and 53% of the cytoplasmic sRNA methyl groups were in guanine derivatives. The nucleolar tRNA methylation enzymes were judged to be true components of the nucleoli and to form a distinct subgroup of the total cellular methylation enzymes. It is suggested that they function as part of a nucleolar protein-synthesizing system.

The presence of an enzyme system for the methylation of endogenous precursor rRNA in isolated nucleoli of rat liver has been described by Culp and Brown (1970) and in isolated nucleoli of Novikoff ascites tumor cells by Liau *et al.* (1970). In contrast to the liver nucleoli, nucleoli isolated from Novikoff ascites hepatoma cells contained an additional group of enzymes which transferred methyl groups from *S*-adenosylmethionine into the nucleotide bases of an exogenous heterologous tRNA, *i.e.*, *Escherichia coli* tRNA. Birnstiel *et al.* (1963) have also demonstrated the ability of heterologous tRNA to stimulate the methylation of RNA by nucleolar preparations from pea seedlings.

The study of tRNA methylases has been a subject of interest because of the essential role that tRNAs and their modified subspecies play in protein synthesis and its regulation (Craddock, 1970), in differentiation (Baliga *et al.* 1965; Pillinger and Borek, 1969; Turkington, 1969), and possibly in malignant transformation (Borek, 1969). These enzymes are strictly specific for the individual bases (Srinivasan and Borek, 1963; Hurwitz *et al.*, 1964) and the pattern and extent of methyl distribution in the tRNA population is specific for species. The specificity appears also to be dependent on the sequence of bases containing the base to be methylated (Baguley and Staehelin, 1969; Baguley *et al.*, 1970; Kuchino and Nishimura, 1970).

Whereas tRNA methylase activity appears predominantly in the soluble fraction of mammalian cells (Burdon *et al.*, 1967; Muramatsu and Fujisawa, 1968; Culp and Brown, 1968), evidence is accumulating that nuclei or nuclear components, particularly nucleoli, show some enzymic activity (Birnstiel *et al.*, 1963; Sirlin *et al.*, 1966; Burdon *et al.*, 1967; Culp and Brown, 1968). Information on these enzymes localized in the nucleus is strikingly lacking; the bulk of study on tRNA methylases of mammalian cells has been conducted on enzymes obtained from the soluble fraction (Rodeh *et al.*, 1967; Kaye and LeBoy, 1968; Gantt and Evans, 1969; Moore and Smith, 1969; Kerr, 1970). The present study demonstrates that isolated nucleoli of Novikoff ascites hepatoma contain a small but significant amount of tRNA methylases, which are easily extractable and have characteristics generally similar to those of enzymes present in the soluble fraction. Evidence is presented to show that the nucleolar enzyme preparation does however methylate the *E. coli* tRNA bases in distinctly dif-

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ferent proportions than does an enzyme preparation from the cell-soluble fraction, and that this pattern results from a discrete group of nucleolar tRNA methylases.

Materials and Methods

[*methyl*-^{3}H]*S*-Adenosyl-L-methionine (1 Ci/mmole) and L-[*methyl*-^{3}H]methionine (2.6 Ci/mmole) was obtained from Schwarz BioResearch and [*methyl*-^{14}C]*S*-adenosyl-L-methionine (47.2 mCi/mmole) from New England Nuclear Corp. *E. coli* B tRNA was purchased from General Biochemicals Inc. and *E. coli* B rRNA was a gift from Mr. P. Gray of this Institute. Yeast tRNA was obtained from Sigma Chemical Corp. Poly(A), poly(I), poly(C), poly(CI), and poly(I)·poly(C) were purchased from P-L Biochemicals, Inc. 1-Methyladenine, N^6-methyladenine, 1-methylguanine, 7-methylguanine, and 5-hydroxymethyluracil were obtained from Sigma Chemical Corp.; 7-methyladenine, N^2-methylguanine, N^2-dimethylguanine, and N^6-dimethyladenine from Cyclo Chemical Corp.; 5-methylcytosine from Nutritional Biochemical Corp.; and thymine from CalBiochem.

Preparation of Nucleoli. Novikoff ascites hepatoma cells were grown in 150–175 g Sprague-Dawley rats from Sprague-Dawley Farms, Madison, Wis. The cells were harvested on days 5 or 6 and washed by suspension in 2 M sucrose–5 mM $MgCl_2$–0.01 M Tris-acetate (pH 7) and centrifugation at 88,700*g* for 15 min. The subsequent isolation of nucleoli was as described elsewhere (Liau *et al.*, 1965; Liau and Hurlbert,[1] 1972). Briefly, the washed tumor cells were suspended in 3 volumes of 0.01 M Tris-acetate (pH 7.0)–5 mM $MgCl_2$–0.2 mM $CaCl_2$ and disrupted by passing through a French pressure cell (American Instrument Co.) at 3000–5000 psi. Sucrose solution (2 M) was added to 0.25 M, and the crude nucleolar fraction was sedimented at 900*g* for 10 min. Nucleoli were purified by suspension of the sediment in 2.2 M sucrose–5 mM $MgCl_2$–0.01 M Tris-acetate (pH 7) and centrifugation at 88,700*g* for 10 min. The nucleolar preparations obtained by this procedure were free of nuclei and nearly free of fibrous and amorphous particles, as judged by phase-contrast microscopy and electron micrographs (Liau and Hurlbert,[1] 1972).

Preparation of Enzyme Extracts. Isolated nucleoli, after being washed twice by centrifugation (600*g* for 5 min) with isotonic sucrose solution (0.25 M sucrose–5 mM $MgCl_2$–0.01 M Tris-acetate, pH 7), were extracted with 10 vol of 0.5 M NH_4Cl–0.5 mM $MgCl_2$–0.01 M dithiothreitol–0.01 M Tris-chloride (pH 7.8) by gentle treatment in a Dounce homogenizer in an ice bath for 10 min. The aggregated materials were removed by centrifugation at 226,000*g* for 30 min in a Spinco Ti 50 rotor. About 95% of the nucleolar tRNA methylases could be solubilized by a single extraction. This solution, containing the nucleolar tRNA methylases, was mixed with an equal volume of glycerol and stored at −20°. The nucleolar residue after this extraction showed very little response to the added *E. coli* tRNA and a decrease of 30–40% of the endogenous rRNA methylating activity was also observed.

Preparations of the cell-soluble, pH 5 enzyme fraction were obtained as a by-product of the preparation of nucleoli (Liau *et al.*, 1965). After cells were disrupted by passage through the French pressure cell, the crude nucleolar fraction was sedimented at 900*g* and the supernatant fraction was then recentrifuged at 226,000*g* for 60 min. The lipid layers were discarded. The clear supernatant (cell soluble or S_{226} fraction) was brought to pH 5 by the addition of 1 N acetic acid and centrifuged at 20,000*g* for 5 min. The pellet, representing about 54–65% of the total activity present in the cell-soluble fractions, was dissolved in 0.5 M NH_4Cl–0.5 mM $MgCl_2$–0.01 M dithiothreitol–0.01 M Tris-chloride (pH 7.8) to give an activity per milliliter equivalent to the nucleolar enzyme preparation, and was centrifuged at 226,000*g* for 30 min to remove insoluble materials. This is designated the cell-soluble, pH 5 preparation and contains not only strictly cytoplasmic soluble enzymes, but also any other enzymes which may be liberated from other cell organelles by the method of cell disruption.

Enzyme preparations were also obtained for comparative studies by direct extraction of whole cells in a Dounce homogenizer at 0° with 10 vol of 0.5 M NH_4Cl–0.5 mM $MgCl_2$–0.01 M dithiothreitol–0.01 M Tris-chloride (pH 7.8).

Assay of tRNA Methylase. The standard assay mixture in a volume of 0.25 ml contained: 0.05 M Tris-acetate (pH 7.8), 0.2 M NH_4Cl, 0.04 M NaF, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP, 50 μg of *E. coli* tRNA (sodium salt), 0.5 μCi of [*methyl*-^{3}H]*S*-adenosyl-L-methionine, and an amount of enzyme preparation containing 70–80 μg of protein (0.05 ml). The incubation was allowed to proceed at 37° for 30 min, unless otherwise stated. The reaction was stopped by immersing the tubes in an ice bath followed by addition of 0.2 mg of bovine serum albumin and 0.25 ml of 0.1 M sodium pyrophosphate. The mixture was precipitated by the addition of one-tenth volume of 4 N perchloric acid. The precipitate was washed twice with 5 ml of cold 0.4 N perchloric acid–1% sodium pyrophosphate. The tRNA was extracted with 1 ml of 2 M NaCl–5 mM NH_2OH at 100° for 30 min. Before heating, the suspension was carefully neutralized with 1 M Tris-chloride (pH 8) using phenol red as indicator. Yeast RNA (0.1 mg) was added to the NaCl extract and the RNA was precipitated by the addition of an equal volume of 10% trichloroacetic acid–2% sodium pyrophosphate. The precipitate was collected on a Millipore filter (0.45 μ) and washed thoroughly with cold 5% trichloroacetic acid–1% sodium pyrophosphate. This washing procedure was necessary to reduce the nonenzymic adsorption of label at zero time to about 0.4 pmole of methyl groups/mg of protein. These controls were determined and subtracted in each experiment.

Analysis of Methylated Products. The method described by Iwanami and Brown (1968) was followed for the identification of methylated bases. Methylated tRNA obtained by extraction with 2 M NaCl was reprecipitated in the presence of 2 mg of carrier yeast RNA with cold 0.4 N perchloric acid and washed twice with cold 0.4 N perchloric acid–1% sodium pyrophosphate. The RNA was hydrolyzed in 1 N HCl at 100° for 1 hr. The hydrolysate was applied to a Dowex 50W-X12 column (0.8 × 15 cm) and the column was eluted with 1 l. of 2 N HCl. The radioactive materials appeared primarily in three fractions, namely, pyrimidine derivatives, guanine, and adenine, which were each collected and evaporated to dryness in a flash evaporator at 37°. The residues were taken up in a small amount of H_2O.

Pyrimidine derivatives were further separated by column chromatography on a Dowex 1-X8 column (0.8 × 15 cm). The column was successively eluted with 120 ml of 0.01 M NH_4Cl and 600 ml of 0.01 N HCl and the fractions were analyzed for ultraviolet light absorption and radioactivity. The radioactive peaks (two nucleoside peaks eluted by 0.01 M NH_4Cl and two mononucleotide peaks eluted by 0.01 N HCl) were each pooled and again were evaporated to dryness. The residues were hydrolyzed to free bases with 35% perchloric acid in sealed tubes saturated with nitrogen gas at 100° for 1 hr. The perchloric acid was neutralized with KOH and

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TABLE I: Distribution of RNA Methylases among Subcellular Fractions.[a]

	% of Total Protein	RNA Methylation: Endogenous RNA pmole/mg of Protein	Endogenous RNA % of Total	*E. coli* tRNA pmoles/mg of Protein	*E. coli* tRNA % of Total	% of Total [^{3}H]tRNA Distribution
Disrupted cell suspension	100	0.21	100	2.05	100	100
Nucleolar pellet	7.1	0.41	11.2	1.25	5.1	1.2
Cell-soluble fraction	22.0	0.72	69.7	7.58	80.1	75.0
Remainder (by difference)	70.9		19.1			23.8

[a] Washed Novikoff ascites tumor cells were disrupted in a French pressure cell. From this disrupted cell suspension were prepared a purified nucleolar pellet and a cell-soluble fraction, as described in Methods. Aliquots of these preparations were taken for the assay of RNA methylase activities, protein, or admixed [^{3}H]tRNA. For the measurement of methylation of endogenous RNA, the assay mixture in a volume of 0.25 ml contained: 0.05 M Tris-chloride (pH 7.8), 0.04 M NaF, 1 mM ATP, 0.5 μCi of [*methyl*-^{3}H]*S*-adenosyl-L-methionine (1 Ci/mmole), and 50 μl of disrupted cell suspension (60–70 μg of protein). For the measurement of methylation of heterologous (*E. coli*) tRNA, the same assay mixture was used in the presence of 0.2 M NH_4Cl and with or without 50 μg of *E coli* B tRNA: the difference between these two values is recorded in the table. After incubation at 30° for 15 min the reaction was stopped and the nucleic acids were extracted as described in Methods. RNA was hydrolyzed in 0.5 ml of 0.3 M KOH at 37° for 18 hr. The alkaline hydrolysate was acidified to 0.2 N $HClO_4$, and the resulting supernatant was then taken for the measurement of radioactivity and RNA content. For an estimation of degree of contamination of nucleoli with components of the soluble fraction, 2.35 mg of [^{3}H]tRNA (31,800 cpm), prepared from tumor cells labeled *in vivo* with [^{3}H]-cytidine, was added to the disrupted cell suspension before fractionation. Data on RNA methylases are the average of two experiments, data on distribution of admixed [^{3}H]tRNA are the result of a separate experiment, and protein data are the average of all three. All data on nucleoli have been corrected for the yield of purified nucleoli, assuming 40% recovery of nucleoli from cells.

the resulting $KClO_4$ precipitate was removed by centrifugation. The supernatant was dried in a desiccator under vacuum and redissolved in a small amount of H_2O.

The bases thus obtained were identified by paper chromatography on Whatman No. 1 in solvent systems described by Dunn and Smith (1958) and by Iwanami and Brown (1968) in the presence of known markers. The solvent of choice for the identification of 1-methyladenine, 5-methylcytosine, thymine, and 5-hydroxymethyluracil was saturated $(NH_4)_2SO_4$–2-propanol–1 M sodium acetate (40:1:9, v/v) descending chromatography for 15 hr, and that for the separation of 1-methylguanine, 7-methylguanine, N^2-methylguanine, and N^2-dimethylguanine was 2-propanol–12 N HCl–H_2O (68:17.6:14.4, v/v) by descending chromatography for 90 hr.

Analytical Procedures. Protein was determined by the method of Lowry *et al.* (1951), RNA by a modified orcinol reaction (Hurlbert *et al.*, 1954), and DNA by the Burton diphenylamine procedure (Burton, 1956).

For the determination of radioactivity by scintillation counting, the dried Millipore filters were immersed in toluene containing 50 g of 2,5-diphenyloxazole and 0.1 g of dimethyl 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter. Bases on Whatman paper were first eluted with 0.5 ml of 0.1 N HCl and counted by mixing this extract with 10 ml of a mixture of the above toluene fluor with methoxyethanol (6:4, v/v). Radioactivity was determined in a Nuclear-Chicago Mark I instrument with corrections for efficiency.

Results

It has been previously reported (Liau *et al.*, 1970) that methylation of RNA by isolated nucleoli could be stimulated 1.5- to 2-fold when *E. coli* tRNA was added to the reaction mixture at low ionic strength. The stimulation of RNA methylation by *E. coli* tRNA became more obvious (4- to 5-fold) when assay was conducted in a medium containing 0.2 M NH_4Cl. When nucleoli were incubated with [*methyl*-^{3}H]*S*-adenosyl-L-methionine plus *E. coli* tRNA, then sedimented by centrifugation at 900*g* for 10 min, the RNA of the nucleolar pellet contained primarily methyl-labeled ribose moieties; that is, the labeled methyl groups were found in the di- and trinucleotide fractions of the alkali-hydrolyzed RNA, indicating methyl substituents on the 2′-OH position of ribose. The sRNA in the supernatant fluid was found to contain labeled methyl groups primarily in the mononucleotides released by alkali, indicating methylation of the purine and pyrimidine bases. The enzyme activity responsible for methylation of the tRNA could be extracted with 0.5 M NH_4Cl–0.5 mM $MgCl_2$–0.01 M dithiothreitol–0.01 M Tris-chloride (pH 7.8). The methylated product of *E. coli* tRNA by the solubilized nucleolar enzymes contained only 6% of the methyl groups as substituents on ribose and 94% as substituents on the bases.

As shown in Table I, the distribution of RNA methylases among the disrupted cell suspension, the nucleolar fraction, and the cell-soluble fraction was studied by analyzing the methylase activity of these fractions obtained after cells had been disrupted by passage through the French pressure cell. The assay was conducted both at low ionic strength to measure activity with endogenous RNA, and in the presence of 0.2 M NH_4Cl, without or with added *E. coli* tRNA, for the determination of methylases capable of acting on heterologous tRNA. The methylases detected in isolated nucleoli without added RNA were primarily methylases acting on endogenous rRNA, whereas those present in the soluble fraction were methylases acting on endogenous tRNA. In Table I, "RNA methylation" (*E. coli* tRNA) refers to only those activities responding to the added *E. coli* tRNA assayed in the presence

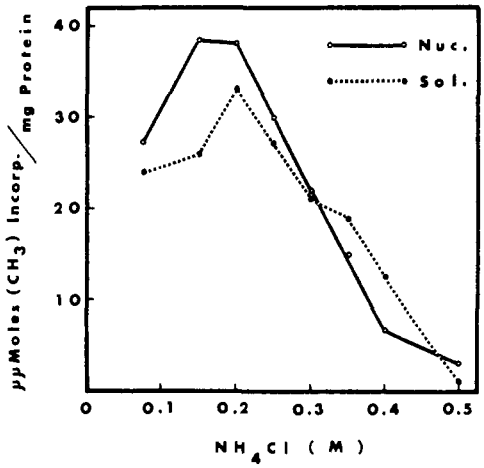

FIGURE 1: Effect of ammonium chloride on the rate of methylation of *E. coli* B tRNA by enzymes from nucleolar and soluble fractions. The reaction mixture (0.25 ml) contained 0.5 μCi of [^{3}H]S-adenosyl-L-methionine (specific activity 1 Ci/mmole), 50 μl of nucleolar enzyme extract or cell-soluble, pH 5 enzyme preparation, NH_4Cl as indicated, and other ingredients as in the standard assay. Incubation was conducted at 37° for 30 min and the incorporation of ^{3}H into a cold perchloric acid precipitable-hot NaCl extractable product was determined as described in Methods.

of 0.2 M NH_4Cl. The endogenous activities obtained under the same assay conditions were subtracted. Isolated nucleoli contained a small but constant and significant proportion of the methylation activity measureable with heterologous tRNA (5%), although the majority of these enzymes was present in the soluble fraction (at least 80%).

The possibility that the tRNA methylase of the nucleolar preparation was merely due to contamination by or adsorption of soluble components was estimated by using [^{3}H]tRNA of Novikoff hepatoma as a marker for the components of the soluble fraction. The labeled RNA was mixed with the homogenate before fractionation. Only about 1% of the admixed [^{3}H]tRNA was found in the nucleolar preparation. The enzyme assay was also performed on an extract of the predominantly chromatin materials, which packed at the top of the tube during the centrifugation of the crude 900*g* nucleolar sediment in 2.2 M sucrose solution. Based on the same packed volume of pellet, the activity of heterologous tRNA methylases from these chromatin materials was only 7% that of nucleoli. Thus, tRNA methylases observed in isolated nucleoli do not appear to result from simple occlusion of components of the soluble fraction.

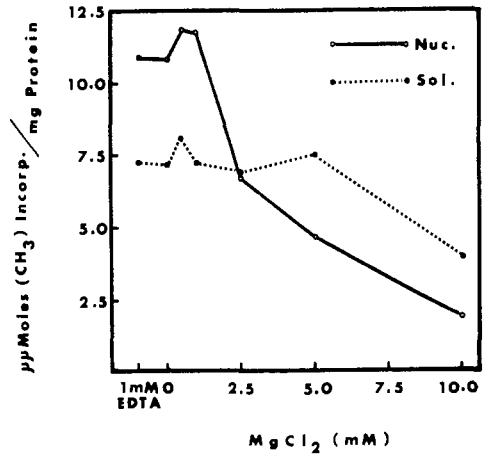

FIGURE 2: Effect of Mg^{2+} on the rate of enzymic methylation of *E. coli* B tRNA. The reaction mixture contained 0.5 μCi of [^{3}H]-*S*-adenosyl-L-methionine, 50 μl of nucleolar enzyme extract or cell-soluble, pH 5 enzyme preparation, EDTA or $MgCl_2$ as indicated, and other ingredients as in the standard assay. Incubation was conducted at 30° for 15 min, followed by assay as described in Figure 1 and Methods.

TABLE II: Comparison of RNAs as Methyl Acceptors for sRNA Methylases.[a]

Substrate	pmoles of CH_3 Incorpd/mg of Enzyme Protein: Nucleolar Enzyme Extract	Cell-Soluble, pH 5 Enzymes
None added	0.23	1.05
E. coli B tRNA	35.7	30.0
E. coli rRNA	0.54	0.84
Yeast tRNA	1.38	2.66
Novikoff tumor tRNA	0.80	1.81
Rat liver tRNA	0.83	0.46
Poly(C)	0.81	1.44
Poly(I)	0.17	0.28
poly(CI)	0.37	1.23
Poly(A)	0.29	0.88
Poly(U)	0.35	0.86
Novikoff tumor nucleolar DNA	0.26	0.77
E. coli tRNA + poly(I)	22.3	13.5

[a] Standard assay conditions were used; see Methods and Figure 1. The reaction mixture contained 0.5 μCi of [^{3}H]-*S*-adenosyl-L-methionine, 50 μl of enzyme preparations, and 50 μg of the various substrates listed.

The nucleolar tRNA methylases could be effectively extracted with 0.5 M NH_4Cl solution as described in Methods. Both the nucleolar extract and the cell-soluble enzyme preparations were relatively stable in this solution, decaying about 2–3% of the activity per day. The enzyme preparations contained protein in the range of 1.3–1.8 mg/ml. In addition, the nucleolar enzyme extracts contained 30–60 μg of RNA/ml, and 6–10 μg of DNA/ml. The cell-soluble enzyme preparation contained 60–90 μg of RNA/ml and no DNA.

Optimal Conditions for Enzyme Activity. Both the nucleolar enzyme extract and the cell-soluble enzymes showed optimal activities at 0.2 M NH_4Cl (Figure 1). A broad pH range (7.5–10) was effective for both enzyme preparations; in fact, pH 9 appeared to be more favorable, particularly for the cell-soluble preparation. However, incubation was routinely carried out at pH 7.8, since *S*-adenosyl-L-methionine has been shown to be unstable at higher pH (Cantoni, 1957), and since appreciable nonenzymatic methylation occurs above pH 8 (Culp and Brown, 1968). The reaction proceeded almost linearly up to 90 min at 37°. The addition of 0.04 M NaF, a known inhibitor of nuclear exoribonuclease (Lazarus and Sporn, 1967), stimulated the nucleolar enzymes by 17% and inhibited the cell-soluble enzymes by 20%. The addition of 1 mM ATP improved both enzyme activities by 17–20%. Figure 2 shows that addition of divalent cations was not required for methylation. On the other hand, the nucleolar enzymes were

TABLE III: Distribution of Labeled Methyl Groups among *E. coli* tRNA Bases after Methylation by Nucleolar Extract or Cell-Soluble Preparations.[a]

	Recovery of ^3H as Per Cent of Total Methylated Bases							
	Nucleolar Extract		Cell Soluble, pH 5		Cell Soluble		Whole Cell	
Methylated bases								
Adenine derivatives	12.3		26.9		23.2		29.4	
1-Methyladenine		11.1		24.5		20.8		27.7
Guanine derivatives	14.8		59.8		60.4		52.4	
1-Methylguanine		1.2		2.7		3.3		4.2
N^2-Methylguanine		6.7		42.4		36.8		31.5
N^2-Dimethylguanine		6.1		12.7		18.0		13.0
7-Methylguanine		0.6		1.2		1.7		1.8
Uracil derivatives	1.7		0.4		0.4		0.5	
Thymine		0.6		0.1		0.1		0.2
5-Hydroxymethyluracil		0.8		0.2		0.2		0.2
Cytosine derivatives	71.3		12.9		16.1		17.8	
5-Methylcytosine		69.3		12.8		15.8		17.4

[a] Enzyme extracts were freshly prepared from Novikoff ascites cells; all extracts were made with 0.5 M NH_4Cl–0.5 mM $MgCl_2$–0.01 M dithiothreitol–0.01 M Tris-chloride (pH 7.8). The "whole cell" preparation was made by stirring washed cells for 10 min at 0° with 10 ml of the NH_4Cl solution per ml of packed cells, followed by centrifugation at 226,000*g* to obtain the supernatant. The cell-soluble and cell-soluble, pH 5 preparations were made after disruption of the washed cells in a french pressure cell and sedimentation of the crude nucleolar fraction. These preparations, and the purification and extraction of nucleoli, are described in Methods. The reaction mixture contained 0.3 mg of *E. coli* B tRNA, and 12.5 μCi of [^{3}H]*S*-adenosyl-L-methionine (sp act. 1 Ci/mmole) in 5 times the amount of the standard assay. The incubation was conducted at 37° for 1 hr. The extent of methylation (^{3}H-methyl groups incorporated per mg of tRNA/hr) under these conditions by nucleolar extract was 0.342 nmole, that by cell-soluble, pH 5 enzymes was 0.360 nmole, that by cell-soluble enzymes was 0.265 nmole, and that by whole cell enzymes was 0.275 nmole. The RNA was prepared for the identification of methylated bases as described in Methods following the procedures of Iwanami and Brown (1968). Data are expressed as per cent of the summed radioactivity recovered in the chromatographed bases. Data on nucleolar extract and cell-soluble, pH 5 enzymes are the average of two experiments. Data on cell-soluble and whole cell enzymes are the result of one experiment each.

progressively inhibited by $MgCl_2$ at concentrations greater than 1.25 mM. The cell-soluble pH 5 enzymes were less sensitive; significant inhibition resulted only at concentrations greater than 5 mM. These experiments determined the best conditions as described in Methods for the methylation of tRNA by the nucleolar enzymes and the same reaction conditions were maintained for a close comparison of the two enzyme preparations.

Substrate Requirements. The results summarized in Table II indicate that of the heterologous RNAs tested only *E. coli* tRNA could function as an effective methyl acceptor, although tRNA from yeast served poorly as methyl acceptor. Homologous tRNAs were almost completely ineffective. Synthetic polynucleotides were ineffective (with the possible exception of poly(C)) or were inhibitory. Poly(I) exhibited the greatest inhibitory action among synthetic polynucleotides tested. DNA failed to serve as methyl acceptor.

Pattern of Methylated Bases. Methylation of *E. coli* tRNA by the nucleolar enzyme extract was compared to methylation by other cellular extracts with regard to the distribution of labeled methyl groups among the purine and pyrimidine bases. The extent of methylation under these conditions (12.5 μCi of [^{3}H]*S*-adenosyl-L-methionine for 1 hr at 37°) by the nucleolar enzymes was 0.34 nmole and that by the cell-soluble, pH 5 enzymes was 0.36 nmole of [^{3}H]CH_3 group incorporated/mg of tRNA per hour. The RNA was processed as described in Methods for the identification of methylated bases. The results are summarized in Table III. All of the methylated bases produced by the cell-soluble, pH 5 enzymes were also detectable in RNA methylated by the nucleolar extract. However, these two enzyme preparations showed a marked difference in the relative degrees of methylation of the four RNA bases. The cell-soluble, pH 5 enzymes caused a high degree of methylation of guanine and adenine (60 and 27%, respectively), while the nucleolar enzymes caused predominantly methylation of cytosine (71%). 1-Methyladenine and 5-methylcytosine were the only methylated bases of adenine and cytosine, whereas four methylated guanines and two methylated uracils were identified. The methylated guanine bases produced by these two enzyme preparations also differed considerably in their relative proportions. The proportions of 1-methylguanine, 7-methylguanine, N^2-methylguanine, and N^2-dimethylguanine produced by the cell-soluble, pH 5 enzymes were about 5, 2, 72, and 22%, respectively, whereas those produced by nucleolar enzymes were 8, 4, 45, and 41%, respectively. Two methylated uracil derivatives were identified as thymine and 5-hydroxymethyluracil.

It seemed possible that the cell-soluble, pH 5 preparation might not be representative of the entire group of cell-soluble methylases. Kerr (1970) noted a specific precipitation of N^2-guanine monomethylase by a pH 5 treatment of rabbit liver extract. The results obtained with the simple high-speed supernatant (S_{226}) extract and the subsequent pH 5 precipitate fractions were, however, quite similar. While the pattern obtained with an extract of whole cells was somewhat different from these two cell-soluble patterns (containing a greater amount of

TABLE IV: Patterns of Methylated Bases of Endogenous 4–6 sRNAs after Labeling *in Vivo* with [*methyl*-^{3}H]Methionine.[a]

Methylated Bases	Recovery of Methyl-^{3}H as Per Cent of Total Methylated Bases			
	Nucleolar tRNA		Cell Soluble RNA	
Adenine derivatives	7.8		16.7	
1-Methyladenine		6.6		15.2
Guanine derivatives	37.9		53.3	
1-Methylguanine		5.9		6.0
N^2-Methylguanine		18.3		33.2
N^2-Dimethylguanine		11.9		10.7
7-Methylguanine		1.9		2.7
Cytosine derivatives	49.8		24.5	
5-Methylcytosine		43.1		21.8
Uracil derivatives	4.5		5.5	
Thymine		3.1		2.1
5-Hydroxymethyluracil		0.6		2.3

[a] Novikoff hepatoma cells were labeled *in vivo* for 3 hr with 250 μCi of L-[*methyl*-^{3}H]methionine (2.6 Ci/mmole) injected intraperitoneally into the rat. The nucleolar and cell-soluble fractions were obtained as described in Materials and Methods. RNAs were prepared from both fractions by extraction with phenol (Liau *et al.*, 1970). The sRNA was purified by treatment with 1.5 M NaCl to precipitate heavy RNA, followed by sucrose density gradient centrifugation to obtain the 4–6S fraction and incubation in 0.5 M Tris-chloride (pH 8.8) at 37° for 1 hr to remove aminoacyl groups. In each case the sRNA was reprecipitated with two volumes of ethanol in the presence of 0.14 M NaCl. Purified RNAs were hydrolyzed with 1 N HCl and methylated bases were analyzed as described in Methods. Substantial incorporation of label from the methyl groups into unmethylated purines was also found, as discussed in the text.

cytosine and adenine derivatives), it too was distinctly different from the pattern obtained with the nucleolar preparation.

The possibility that the differing nucleolar and cell-soluble, pH 5 patterns might result from influence by some factor present in the preparations was tested by mixing the two preparations. The resulting specific activity and pattern of labeled methylated bases (data not shown) was the average of the results of the two preparations run separately. Thus, the observed difference in the patterns of methylated bases by these two enzyme preparations must represent intrinsic differences in the enzyme species involved. Nor can this difference be attributed to difference in ribonuclease content of the two enzyme preparations, since neither enzyme preparation caused a loss of cold perchloric acid-precipitable radioactivity when [^{3}H]tRNA of Novikoff hepatoma was incubated in the same reaction mixture at 37° up to 1 hr.

Labeling of Endogenous RNA by [^{3}H]Methionine in Vivo. To further examine these findings, the *in vivo* labeling patterns of 4–6S RNA of the cell-soluble fraction and the nucleolar fraction were analyzed after the tumor cells were labeled *in vivo* with L-[*methyl*-^{3}H]methionine for 3 hr. Soluble RNA's were prepared from both fractions, treated to free them from other species of RNA, and then subjected to the same analysis as illustrated in Table III. The results are shown in Table IV. When RNA was labeled *in vivo* with [*methyl*-^{3}H]methionine, label was also incorporated into the purine skeleton in addition to the methylation of RNA bases. When the cell-soluble sRNA was analyzed, 15.5% of the radioactivity in the guanine fraction and 30.8% of the radioactivity in the adenine fraction from Dowex 50 were found on subsequent paper chromatography to be in unmethylated guanine and adenine, respectively. The corresponding values due to purine ring synthesis in nucleolar tRNA were more substantial, amounting to 43.5% of the guanine peak and 81.8% of the adenine peak. The data shown in Table IV refer to distribution of labels among the methylated bases only, and have not been corrected for the contribution due to purine ring synthesis; the correction factors should, however, be small. The patterns of bases methylated *in vivo* are considerably different for these two sRNA preparations, again showing similar trend of greater cytosine methylation by nucleolar enzymes and greater guanine methylation by cell-soluble enzymes. The patterns of methylated bases differed from those produced by methylation *in vitro* of *E. coli* tRNA by the two enzyme preparations, as might be expected from the work of Iwanami and Brown (1968) and Culp and Brown (1968) who compared the action of methylases in endogenous tRNA *in vivo* and heterologous tRNA *in vitro*.

Specificity of Methylation. It is well known that the specificity of tRNA methylases resides not only with the specific base but also with the sequence where this specific base is located. Therefore it was believed that analysis of methyl groups in oligonucleotide fragments released from tRNA by ribonuclease would provide insight into the specificity of different enzyme preparations.

E. coli tRNA was methylated by nucleolar enzymes with [^{14}C]*S*-adenosyl-L-methionine or by cell-soluble, pH 5 enzymes with [^{3}H]*S*-adenosyl-L-methionine. The RNA's were mixed and subjected to digestion by pancreatic ribonuclease, and chromatography of oligonucleotides was conducted as described by Tener (1967). Most of the radioactive nucleotides were distributed among the mononucleotide through tetranucleotide peaks. ^{3}H:^{14}C ratios were variable among these radioactive peaks, ranging from lowest in the mononucleotide peak (2.4) to highest in one of the dinucleotide peaks (11.4) and one of the trinucleotide peaks (10.8). A comparable variation in ^{3}H:^{14}C ratios among radioactive oligonucleotides (with a different pattern) was obtained in separate experiments when RNA was digested with T_1 RNase. These results indicated that the nucleolar and cell-soluble, pH 5 enzymes were each methylating specific sequences, rather than distributing the methyl groups randomly. The question remained whether the observed difference in methylation patterns results from an exclusive difference in specificity between two sets of enzyme species or from a difference in the relative proportions of a common set of species of tRNA methylating enzymes.

If an exclusive difference in specificity exists between the two enzyme preparations, then saturation of available sites of methylation by one enzyme preparation should not interfere with the methylation by another enzyme preparation. The experiments shown in Figure 3 were therefore designed to use a limited amount of tRNA with an excess amount of one enzyme preparation and [^{3}H]*S*-adenosyl-L-methionine to saturate the available sites of methylation followed by addition of the other enzyme preparation to determine whether additional incorporation of methyl groups could occur. Saturation was achieved by 0.3 mg of the nucleolar enzymes within 30

min of incubation at 37° with an incorporation of 16.2 pmoles of methyl-^{3}H groups. Addition of 0.1 mg more of nucleolar enzymes at 30 min (Figure 3a) caused no additional incorporation during a subsequent 45-min incubation. Addition of 0.16 mg of cell-soluble enzymes caused a significant additional incorporation of methyl-^{3}H groups (Figure 3a), and 8.7–10 pmoles could be further incorporated during the 45-min incubation.

When the cell-soluble enzyme preparation was employed first, it was difficult to achieve saturation of the sites of methylation within a short time period. With a seemingly great excess of enzymes, 1 mg, the incorporation of methyl groups began to level off after achieving an incorporation of 34.6 pmoles of methyl-^{3}H by incubation at 37° for 45 min; apparently some of the methylating enzymes were present in small proportions. The addition of 0.1 mg of nucleolar enzymes at this time caused a slight but barely significant additional incorporation over a control containing no additional enzyme (Figure 3b). It appears, then, that the cell-soluble enzyme preparation contains most, if not all, of the enzyme species present in nucleoli, and that the nucleolar preparation contains a limited and discrete fraction of the total number of cellular tRNA methylating enzymes.

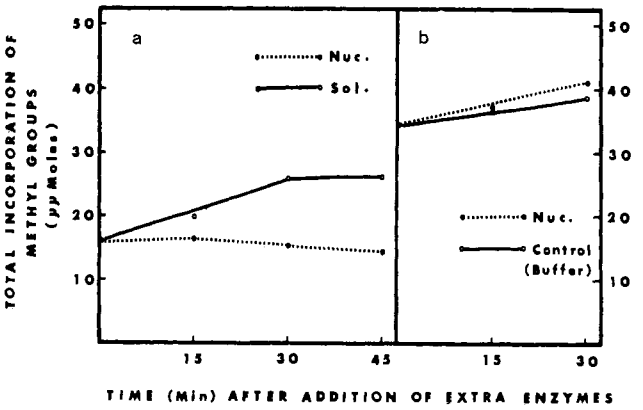

FIGURE 3: Effect of presaturation of the sites of methylation by one enzyme preparation on the activity of a second enzyme preparation. (a) Each reaction mixture contained 7 μg of *E. coli* B tRNA, 0.3 mg of nucleolar extract protein, and 0.18 mM [^{3}H]*S*-adenosyl-L-methionine (specific activity 17.6 mCi/mmole) in twofold of the standard assay. The reaction was conducted at 37° for 30 min; 16.2 pmoles of CH_3 were incorporated. To each of one set of reaction tubes, 0.1 mg of additional nucleolar enzyme protein was added; to the other set, 0.16 mg of cell-soluble pH 5 enzyme protein was added, and the reaction was continued for the time period indicated. (b) Each reaction mixture contained 7 μg of *E. coli* B tRNA, 1 mg of cell-soluble pH 5 enzyme protein, and 0.18 mg of 1 mM [^{3}H]*S*-adenosyl-L-methionine (specific activity 17.6 mCi/mmole) in twofold the standard assay. The reaction was conducted at 37° for 45 min. 34.6 pmoles were incorporated. To each tube of one set, 0.1 mg of nucleolar extract protein was added; to each tube of the other set, the same volume of buffer used for the enzyme preparation was added, and the reaction was continued for the time period indicated.

Discussion

The results presented here show a small but consistent and reproducible content of tRNA methylases in nucleoli isolated from the Novikoff ascites rat tumor. These nucleoli have been extensively purified, studied, and characterized with regard to synthesis of rRNA (Liau and Hurlbert,[1] 1972; Hurlbert *et al.*, 1966; Hurlbert *et al.*, 1969) and are nearly free of particulate and occluded soluble components (see footnote to Table I). It is, however, difficult to preclude the possibility that soluble cytoplasmic methylases are in some way adsorbed to the nucleoli as an artifact of the preparation, and this point will be discussed further.

The major point confirming localization of tRNA methylases in nucleoli is the unique pattern of bases methylated by these enzymes. The activity of tRNA methylases and the patterns of methylated bases are, however, often influenced by natural inhibitors present in the cellular extract (Kerr, 1970; Rodeh *et al.*, 1967; Kaye and LeBoy, 1968). The enzyme preparations and assay conditions (*e.g.*, high ionic strength) employed in this study were intended to avoid the effects of these natural inhibitors known to be present in relatively low amounts in extracts of Novikoff ascites cells (Kerr, 1970, 1971). Furthermore, the nucleolar extract was prepared by a method parallel to the methods of preparing total or cell-soluble extracts. The pattern of bases methylated by the cell soluble, pH 5 enzyme preparation resembled that obtained with an extract of HeLa cells (Culp and Brown, 1968), *i.e.*, methylated guanines predominated. The nucleolar enzymes caused a preferential methylation of cytosine and distinctly different proportions of N^2-methyl- and N^2-dimethylguanines. Further confirmatory evidence for a distinctive methylation system in nucleoli is provided by the observation in Table IV that the *in vivo* methylation patterns of endogenous RNAs tend to resemble those caused by the extracted enzymes acting on heterologous tRNA. Methylases were not adsorbed by the chromatin fraction during preparation of nucleoli. It is therefore unlikely that the tRNA methylases detected in the isolated nucleoli are merely a result of contamination from soluble fraction.

The differences observed in patterns of bases methylated by these two enzyme preparations are due to differences in the relative amounts of the various types of enzymes methylating tRNAs; whether the nucleolus contains any unique *species* of tRNA methylases cannot be judged at present. The nucleolar extract does not contain all of the enzyme species present in the cell-soluble preparation, since the cell-soluble enzymes did induce further methylation of a substrate which had already been saturated by methyl groups from the nucleolar enzymes (Figure 3). On the other hand, the cell-soluble preparation did include all of the nucleolar enzymes, a result which does not exclude the possibility that some of the nucleolar enzyme species are unique, since the process of cell disruption probably destroys and extracts some of the nucleoli.

Muramatsu and Fujisawa (1968) detected very little methyl-labeled tRNA in liver nucleoli and Culp and Brown (1970) did not detect tRNA methylases in isolated nucleoli of rat liver. Birnstiel *et al.* (1963) indicated the presence of tRNA methylases in isolated nucleoli of pea seedlings, and Sirlin *et al.* (1966) showed cytochemical evidence for methylation of tRNA in nucleoli of *Smittia*. The preparation of nucleoli and assay conditions in the studies by Culp and Brown and Birnstiel *et al.* were different from ours. For instance, our experience indicates that sonication methods for the preparation of nucleoli are more likely to extract some nucleolar components than does the French pressure cell method, and tumor tissues are known to be more active in methylation than liver tissue. Resolution of these seemingly controversial results will require comparative studies with different species and procedures, preferably with homologous under-methylated tRNA as substrate.

The presence of tRNA methylases in nucleoli might not be anticipated. It has been well established that transfer RNA is made on a DNA template in the nucleus (Perry, 1962; Chip-

chase and Birnstiel, 1963; Cook *et al.*, 1964; Woods and Zubay, 1965). Subsequent modification, including change in size and base alterations, is believed to take place in the cytoplasm (Mowshowitz, 1970) and is more closely correlated with the process of protein synthesis (Tidwell, 1970) than to the synthesis of tRNA. Modification of tRNA bases has been implicated as essential in the recognition of a specific codon (Capra and Peterkofsky, 1968), and in the binding of aminoacyl-tRNA to ribosomes (Gefter and Russell, 1969). Isolated nucleoli have, however, been shown to contain transfer RNA (Nakamura *et al.*, 1968) and amino acid activating enzymes (Brandt and Finamore, 1963; Lamkin and Hurlbert,[2] 1972) and to carry out protein synthesis (Birnstiel and Hyde, 1963; Zimmerman *et al.*, 1969; Izawa and Kawashima, 1969; Lamkin and Hurlbert,[1] 1972). The activity of tRNA methylases in nucleoli may then be specifically coordinated with these protein synthetic functions of nucleoli.

References

Baguley, B. C., and Staehelin, M. (1969), *Biochemistry 8*, 257.

Baguley, B. C., Wehrli, W., and Staehelin, M. (1970), *Biochemistry 9*, 1645.

Baliga, B. S., Srinivasan, P. R., and Borek, E. (1965), *Nature (London) 208*, 555.

Birnstiel, M. L., Fleissner, E., and Borek, E. (1963), *Science 142*, 1577.

Birnstiel, M. L., and Hyde, B. B. (1963), *J. Cell Biol. 18*, 41.

Borek, E. (1969), Exploitable Molecular Mechanisms and Neoplasia, Baltimore, Md., Williams and Wilkins, 163.

Brandt, E. E., and Finamore, F. J. (1963), *Biochim. Biophys. Acta 68*, 618.

Burdon, R. H., Martin, B. T., and Lal, B. M. (1967), *J. Mol. Biol. 28*, 357.

Burton, K. A. (1956), *Biochem. J. 62*, 315.

Cantoni, G. L. (1957), *Methods Enzymol. 3*, 600.

Capra, J. D., and Peterkofsky, A. (1968), *J. Mol. Biol. 33*, 591.

Chipchase, M. I. H., and Birnstiel, M. L. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 692.

Cook, R. A., Bouchard, J. P., and Fraser, M. J. (1964), *Can. J. Biochem. 42*, 859.

Craddock, V. M. (1970), *Nature (London) 228*, 1264.

Culp, L. A., and Brown, G. M. (1968), *Arch. Biochem. Biophys. 124*, 483.

Culp, L. A., and Brown, G. M. (1970), *Arch. Biochem. Biophys. 137*, 222.

Dunn, D. B., and Smith, J. D. (1958), *Biochem. J. 68*, 627.

Gantt, R., and Evans, V. J. (1969), *Cancer Res. 29*, 536.

Gefter, M. L., and Russell, R. L. (1969), *J. Mol. Biol. 39*, 145.

Hurlbert, R. B., Liau, M. C., and Miller, E. G. (1966), *Proc. Int. Symp. Cell Nucl. Metab. Radiosensitivity*, 231.

Hurlbert, R. B., Miller, E. G., and Vaughan, C. L. (1969), *Advan. Enzyme Regul. 7*, 219.

Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R. (1954), *J. Biol. Chem. 209*, 23.

Hurwitz, J., Gold, M., and Anders, M. (1964), *J. Biol. Chem. 239*, 3462.

Iwanami, Y., and Brown, G. M. (1968), *Arch. Biochem. Biophys. 124*, 472.

Izawa, M., and Kawashima, K. (1969), *Biochim. Biophys. Acta 190*, 139.

Kaye, A. M., and LeBoy, P. S. (1968), *Biochim. Biophys. Acta 157*, 289.

Kerr, S. J. (1970), *Biochemistry 9*, 690.

Kerr, S. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 406.

Kuchino, Y., and Nishimura, S. (1970), *Biochem. Biophys. Res. Commun. 40*, 306.

Lazarus, H. M., and Sporn, M. B. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1386.

Liau, M. C., Flatt, N. C., and Hurlbert, R. B. (1970), *Biochim. Biophys. Acta 224*, 282.

Liau, M. C., Hnilica, L. S., and Hurlbert, R. B. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 626.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Moore, B. G., and Smith, R. C. (1969), *Can. J. Biochem. 47*, 561.

Mowshowitz, D. B. (1970), *J. Mol. Biol. 50*, 143.

Muramatsu, M., and Fujisawa, T. (1968), *Biochim. Biophys. Acta 157*, 476.

Nakamura, T., Prestayko, A. W., and Busch, H. (1968), *J. Biol. Chem. 243*, 1368.

Perry, R. P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 2179.

Pillinger, D., and Borek, E. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1145.

Rodeh, R., Feldman, M., and Littauer, U. Z. (1967), *Biochemistry 6*, 451.

Sirlin, J. L., Jacob, J., and Birnstiel, M. L. (1966), *Nat. Cancer Inst. Monogr. 23*, 255.

Srinivasan, P. R., and Borek, E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 529.

Tener, G. M. (1967), *Methods Enzymol. 12*, 398.

Tidwell, T. (1970), *J. Cell Biol. 46*, 370.

Turkington, R. W. (1969), *J. Biol. Chem. 244*, 5140.

Woods, P. S., and Zubay, G. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1705.

Zimmerman, E. F., Hackney, J., Nelson, P., and Arias, I. M. (1969), *Biochemistry 8*, 2636.

[2] Manuscript in preparation.