slightly outside the experimental error, however, whereas according to mechanism III, the presence of the heme-linked proton increases the binding specific rate constant by a factor of 10^4, a remarkable catalytic effect. Values of k_{1app} for fluoride binding by metmyoglobin are much smaller, ranging from 2×10^{-1} to 5×10^{-2} M^{-1} sec^{-1} over the pH range 6–9 (Blank *et al.*, 1961).

Acknowledgments

We thank Mr. William Randall for running the computer programs and Mr. Aniruddh Hathi for technical assistance.

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Properties of Soluble Ribonucleic Acid Methylases from Rat Liver*

Ruth Rodeh, Michael Feldman, and Uriel Z. Littauer

ABSTRACT: The properties of a purified enzyme fraction from rat liver, which catalyzes the transfer of methyl group from *S*-adenosyl-L-methionine to soluble ribonucleic acid (sRNA), were examined. The reaction was found to be stimulated by 0.25 M ammonium ions. The RNA methylase(s) from rat liver are not active in some heterologous systems, and, therefore, seem to be highly specific in their methyl acceptor requirements. The products of the methylation reaction were identified as 5-methylcytosine, 1-methyladenine, thymine, 7-methylguanine, and N^2-methylguanine (or N^2-dimethylguanine). No differences were found in the specific activities of crude sRNA methylase(s) from normal or regenerating rat liver, nor was there any difference in the extent and pattern of methylation during liver regeneration. The RNA methylase(s) are inhibited by the presence of deoxyribonucleic acid (DNA) and synthetic polynucleotides.

Soluble ribonucleic acid from rat liver, as well as sRNA from other sources, contains a number of methylated purine and pyrimidine bases, in addition to the four common bases (Smith and Dunn, 1959; Dunn, 1959, 1961; Sluyser and Bosch, 1962; Price *et al.*, 1963). sRNA isolated from any given source is characterized by the number and type of the methylated bases it contains. The methyl groups are incorporated at the macromolecular level by enzymes which catalyze the transfer of methyl groups from SAM[1] to sRNA. These enzymes can be detected in many organisms including rat liver homogenates (Srinivasan and Borek, 1963). The sRNA methylases are species specific, and consequently, sRNA which is fully saturated with respect to its homologous enzymes offers new sites for

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[1] Abbreviations used: CTAB, cetyltrimethylammonium bromide; DTE, dithiothreitol; SAM, *S*-adenosyl-L-methionine; SAE, *S*-adenosyl-L-ethionine; TCA, trichloroacetic acid.

methylation by heterologous sRNA methylases (Svensson *et al.*, 1963; Srinivasan and Borek, 1963; Gold *et al.*, 1963; Birnstiel *et al.*, 1963). Here we present some of the properties of sRNA methylase(s) from rat liver.

Materials and Methods

[Methyl-^{14}C]SAM was obtained from Tracerlab. [1-Ethyl-^{14}C]SAE was purchased from New England Nuclear Corp. L-[Methyl-^{14}C]methionine was obtained from the Radiochemical Center, Amersham, England. L-[^{14}C]Phenylalanine and L-[^{3}H]phenylalanine were obtained from Schwartz Laboratories, Inc. SAM was obtained from Sigma Chemical Co. and purified by chromatography on Whatman No. 3 paper in an ethanol–H_2O–1 N acetic acid (63:34:1) system. DTE (Cleland's reagent) was purchased from Calbiochem (Cleland, 1964). Calcium phosphate gel was obtained from Sigma Chemical Co.

Escherichia coli sRNA was prepared according to the procedure of Zubay (1962). For the preparation of *Micrococcus lysodeikticus* sRNA, 5 g of spray dried cells (Miles Chemical Co.) was suspended in 100 ml of 0.001 M EDTA, pH 8.0, and incubated with 50 mg of lysozyme for 10 min at 4°. To this suspension 100 ml of 75% phenol was added and the suspension was stirred for 1 hr at 20°. The suspension was then cooled to 4°, centrifuged, and to the upper aqueous layer ethanol (2.2 volumes) and 5 M NaCl (0.2 volume) were added. After 12 hr at −15° the precipitate was collected by centrifugation, extracted with 5 ml of 1 M NaCl at 0°, and precipitated with two volumes of ethanol at −15°. Rat liver sRNA was isolated directly from the 105,000*g* cytoplasmic supernatant of liver homogenate by treatment with a phenol–water mixture (Daniel and Littauer, 1963). Chick and calf liver sRNA were prepared as previously described (Daniel and Littauer, 1963). Yeast sRNA was prepared according to the procedure of Monier *et al.* (1960). Wheat germ sRNA was kindly obtained from Dr. C. A. Dekker. *E. coli* ribosomal RNA was prepared according to a previously described procedure (Littauer and Eisenberg, 1959) and *M. lysodeikticus* DNA was prepared according to the procedure of Marmur (1961).

Polyadenylic, polycytidylic, polyinosinic, and polyuridylic acids were obtained from Miles Chemical Co. Polyguanylic, poly UC, and poly UAG, were supplied by Dr. Y. Kimhi and prepared by means of purified *E. coli* polynucleotide phosphorylase (Y. Kimhi and U. Z. Littauer, in preparation). 1-Methyladenine was purchased from Sigma Chemical Co. 6-Methyladenine, 6-dimethyladenine, 5-methylcytosine, thymine, and 3-methylcytidine were obtained from Calbiochem. 3-Methylcytosine was prepared by formic acid hydrolysis of 3-methylcytidine. 1-Methylguanine, 2-methylguanine, 2-dimethylguanine, and 7-methylguanine were kindly supplied by Dr. G. B. Elion.

Preparation of Crude Extract. Albino male rats (2-months old) were killed by cervical fracture, and the livers were quickly excised and placed in solution A (0.25 M sucrose–0.01 M $MgCl_2$–0.01 M Tris buffer, pH 8.0, and 0.25 mg/ml of DTE), at 0°. Subsequent steps were carried out at 0–4°. The livers were minced with scissors and, after repeated washings with solution A, homogenized with two volumes of solution A. The homogenate was centrifuged at 18,000*g* for 10 min to remove cell debris and the supernatant solution was then centrifuged at 105,000*g* for 1 hr. The clear cytoplasmic supernatant fluid was drawn off with a pipet. The supernatant solution thus obtained was diluted with solution A to a protein concentration of 10 mg/ml, dialyzed for 3 hr against 0.01 M Tris buffer, pH 8.0, and then 0.1 volume of DTE solution (2.5 mg/ml) was added. (Whenever the next step of purification was followed, the dialysis step was omitted.)

Preparation of Enzyme Fraction. To the supernatant fraction, saturated ammonium sulfate solution (at 4°) was added, with rapid stirring over a 5-min interval, to 29% saturation. The stirring was continued for 30 min after addition of ammonium sulfate, and the precipitate was removed by centrifugation. Saturated ammonium sulfate solution was added to the supernatant solution with rapid stirring over a 5-min interval, to attain 42% saturation. The mixture was stirred for 30 min and centrifuged at 18,000*g* for 10 min. The supernatant fluid was discarded and the precipitated protein was dissolved in a minimal volume of 0.01 M Tris buffer, pH 8.0, and dialyzed for 3 hr against the same buffer. The ammonium sulfate fraction was diluted with 0.01 M Tris buffer, pH 8.0, to a protein concentration of 20 mg/ml.

Calcium phosphate gel suspension (3.5 ml) (10.1% dry material) was centrifuged and the supernatant fluid was discarded. The dialyzed ammonium sulfate fraction (10 ml) (20 mg/ml) was thoroughly mixed with the gel, and after 10 min the mixture was centrifuged for 20 min at 10,000*g*. The supernatant fluid was discarded and the gel precipitate was mixed with 10 ml of 0.4 M potassium phosphate buffer, pH 8.0. The suspension was centrifuged immediately and the supernatant solution was collected and dialyzed for 4 hr against 0.01 M Tris buffer, pH 8.0. DTE (one-tenth volume) (2.5 mg/ml) was added to the dialyzed protein solution which was then diluted with 0.01 M Tris buffer, pH 8.0, containing 0.25 mg/ml of DTE, to a protein concentration of 5 mg/ml and stored at −15°.

Enzyme Assay. Each incubation mixture (0.3 ml) contained 25 μmoles of Tris buffer, pH 9.0 (the final pH is 8.8), 80 μmoles of ammonium acetate, 5–40 μg of *E. coli* sRNA, 25 μg of DTE, various amounts of [methyl-^{14}C]SAM, and enzyme as indicated. After incubation at 37° for 60 min, the reaction was stopped by immersing the tubes in an ice bath; 0.05 ml of carrier sRNA solution (10 mg/ml) was added, and the sRNA was precipitated with 0.05 ml of 0.5% CTAB solution at 4° (Jones, 1953). The precipitate was washed twice with 2.5 ml of cold water. The sRNA was extracted with 1 ml of 2 M NaCl solution, and precipitated by addition of 2.5 volumes of cold 96% ethanol. The samples were kept at −15° for 60 min, and the sRNA was centrifuged out and dissolved in 0.5 ml of H_2O.

A 0.1-ml aliquot was taken to measure radioactivity (with a Nuclear-Chicago gas-flow counter), and the other portion to measure the distribution of [^{14}C]-methyl groups among the nucleotides. This method was found to give lower control values (80–100 cpm) than the trichloroacetic acid precipitation method (1000–3000 cpm). In addition, the control values with the CTAB method were not affected by the protein concentration in the assay system. One unit of enzyme was defined as that amount catalyzing the incorporation of 1 mμmole of methyl group into sRNA in 1 hr at 37°.

The rate of methylation was found to be directly proportional to the enzyme concentration up to 0.8 mg of protein/reaction mixture. When extent of reaction was measured, the reaction mixture contained 5 μg of RNA and 1–2 mg of protein. Under these conditions, longer incubation or addition of more enzyme did not increase the extent of incorporation.

Identification of Methylated Nucleotides in sRNA. sRNA was hydrolyzed to the mononucleotide level by incubation with 1 N $HClO_4$ at 28° for 18 hr. The acid was neutralized by adding 4 N KOH, and the resulting $KClO_4$ precipitate was removed by centrifugation and washed twice with 0.5 ml of H_2O. To remove excess of K^+, Dowex 50-H^+ was added to the combined supernatant with phenolphthalein as an internal indicator. The Dowex was removed by centrifugation, washed twice with 0.5 ml of H_2O, and then with 0.5 ml of 0.01 N NH_4OH. The combined supernatants were concentrated to dryness and the nucleotides were then dissolved in 25 μl of 10% acetic acid adjusted to pH 2.8 with concentrated NH_4OH solution. In some experiments sRNA was hydrolyzed by incubation with 0.4 M KOH at 37° for 18 hr. The K^+ ions were removed by the addition of Dowex 50-H^+ and the procedure was continued as described above. Electrophoresis was carried out in 10% acetic acid adjusted to pH 2.8 on Whatman 3MM paper (Ingram and Pierce, 1962). The paper was dried, cut into small strips, and counted in a liquid scintillation counter. Hydrolysis of nucleotides to free bases was carried out by treatment with 70% formic acid at 175° for 35 min. The bases thus obtained were subjected to descending chromatography on Whatman paper No. 1 in isopropyl alcohol–H_2O–NH_4OH (85:15:1.3) for 18 hr, in the presence of known markers. The paper was then dried and cut into small strips and counted in a liquid scintillation counter (Packard Tri-Carb liquid scintillation spectrometer).

Partial Hepatectomy. Surgery was performed under ether anesthesia. Partial hepatectomy refers to the removal of approximately 67% of the liver (median and left lateral lobes).

Results

Properties of the Enzyme Fraction. The specific activity and extent of methylation of the crude extract to that of the enzyme fraction is compared in Table I. The specific activity of the enzyme fraction is increased fivefold by the purification procedure. It should also be noted that the per cent of total radioactivity found in guanylic acid was increased twofold. In addition the table shows that the extent of methylation is slightly improved by the purification procedure. The enzyme units yield in the purification procedure is about 20%. The instability of sRNA methylase(s) has handicapped any further purification. The sRNA methylase(s) from rat liver was found to be unstable; about 50% of its activity was lost on several days storage at 0° or upon freezing at −15°. Attempts to further purify the enzyme mixture or to separate the different enzymes from each other were unsuccessful, and resulted in a complete loss of activity. In addition, different batches of enzyme preparations varied in their activities. The nucleolytic activity of the purified fraction is low, since upon incubation with this fraction, sRNA looses only 10% of its phenylalanine-accepting activity (Table II). It should be noted that in this experiment unfractionated sRNA was used; therefore, one cannot determine the effect of methylation on the acceptor function of phenylalanine specific sRNA chains. To test this effect it will be necessary to use a highly purified phenylalanine sRNA preparation.

Effect of pH on the Rate of Reaction. In experiments not illustrated here, the rate of the methylation reaction was measured in the range of pH 7.2–9.2. The optimal

TABLE I: Comparison of sRNA Methylase(s) Activity before and after Purification.[a]

Enzyme	Sp Act. (mμ-moles/60 min mg of protein)	Extent of Methylation (mμmoles/mg of RNA	Total Units	Pattern of Methylation[b] (% of total radioactivity)		
				C + A	G	U
Crude extract	0.062	12.0	66	60	35	5
Enzyme fraction	0.33	15.0	13	34	63	3

[a] Standard assay conditions were used (see Methods). The reaction mixture contained 0.5 μc of [methyl-^{14}C]SAM (sp act. 29.9 mc/mM, 22,400 cpm/mμmole), and 5 μg of normal *E. coli* sRNA. [b] The values are obtained under conditions where the extent of reaction was measured. The products of the reactions were hydrolyzed with 1 N $HClO_4$ and then subjected to paper electrophoresis. The following abbreviations have been used in the table: C + A, cytidylic acid plus adenylic acid; G, guanylic acid; U, uridylic acid.

TABLE II: Effect of sRNA Methylase on Phenylalanine sRNA Acceptor Ability.

Pretreatment of sRNA	[^{14}C]Phenylalanine Incorpd[a] (cpm/mg of sRNA
No treatment	51,000
Incubation with the enzyme fraction[b]	46,000

[a] The reaction mixture for incorporation of L-[^{14}C]-phenylalanine was as follows: 0.3 μmole of adenosine triphosphate, 46 μmoles of Tris buffer (pH 7.8), 0.15 μmole of reduced glutathionine, 1.6 μmoles of $MgCl_2$, 100 μg of normal *E. coli* sRNA, 0.2 μc of L-[^{14}C]-phenylalanine (sp act. 0.36 μc/mμmole), and 20 μg of purified phenylalanine synthetase (Bergmann *et al.*, 1961). The reaction mixture (0.07 ml) was incubated for 30 min at 37°. The RNA was precipitated with 5% TCA and filtered onto Millipore membranes. [b] The RNA (100 μg) was preincubated with the purified enzyme fraction (20 mg) and unlabeled SAM as described in Methods. The reaction mixture was cooled to 4° and extracted with 90% phenol. The RNA was precipitated with ethanol and dialyzed against 0.005 M NaCl solution.

pH was found to be 8.8 for methylation of guanine as well as for adenine plus cytosine.

Effect of Ions on the Rate of Reaction. As shown in Figure 1, making the reaction mixture 0.25 M in NH_4^+ ions increased the rate of methylation threefold. Excess of NH_4^+ ions was, however, inhibitory. The rate of methylation of all the four bases was increased to the same extent. Mg^{2+} ions had no effect on the rate of the reaction.

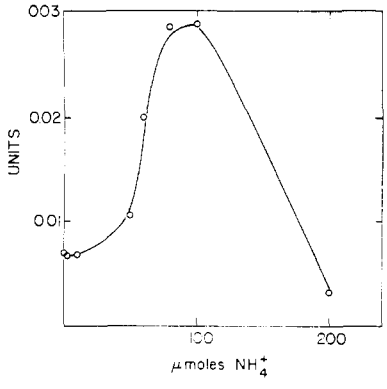

FIGURE 1: Effect of ammonium ions on the rate of enzyme methylation of normal *E. coli* sRNA. The reaction mixture (0.35 ml) contained 0.3 μc of [methyl-^{14}C]SAM (sp act. 26.6 mc/mM, 19,000 cpm/mμmole), 0.5 mg of enzyme fraction, and NH_4Cl as indicated.

Specificity of the Methylation Reaction to SAM. Ethyl groups derived from [ethyl-^{14}C]SAE were not found to be incorporated into sRNA, even when the [ethyl-^{14}C]SAE concentration was increased up to 0.12 μmole/reaction mixture. In contrast, [^{14}C]methyl was incorporated into sRNA even at low SAM concentration (0.01 μmole).

Species Specificity of the Enzyme Fraction. sRNAs isolated from various sources were examined for their ability to serve as acceptors with the rat liver enzyme. Table III contains data on the extent and pattern of methylation of the different RNAs tested. Bacterial sRNAs were methylated significantly while yeast sRNA served as a poor methyl acceptor for the rat liver enzyme(s). Wheat germ sRNA could not be methylated, nor could RNAs from even closer origins such as chick or calf liver sRNA. Apparently, not all the heterologous sRNAs can be methylated. One may postulate that the enzyme from rat liver requires a very specific site or sites on the RNA chain for methylation. Such sites are probably free of methyl groups in the bacterial sRNA and absent or saturated with methyl groups in the other RNAs which were tested. It is also interesting to note that the pattern of methyl nucleotides did not vary significantly for *E. coli* and *M. lysodeikticus* sRNA. Moreover, it was observed that normal *E. coli* sRNA and "methionine-starved" *E. coli* sRNA did not show a significant difference in their methyl group acceptor ability. *E. coli* "methionine-starved" s-RNA is a good methyl acceptor for the homologous *E. coli* sRNA methylases while the normal s-RNA is not. On the other hand, the "methionine-starved" sRNA is only a slightly better substrate for the rat liver enzyme than is the normal species. The pattern of methyl nucleotides is essentially the same, except that "methionine-starved" sRNA accepts more methyl groups into uridine than does normal *E. coli* sRNA.

Synthetic polynucleotides, *E. coli* rRNA, and *M. lysodeikticus* DNA all failed to serve as methyl group acceptors. However, *M. lysodeikticus* DNA, poly A, poly C, poly I, and poly G were found to inhibit the methylation reaction of *E. coli* sRNA (Table IV). In all these cases there was no change in the pattern of methyl nucleotides, even when added DNA caused 50% inhibition. According to this finding, experiments using crude extracts or unpurified nucleic acid preparations may lead to inaccurate results.

Identification of the Reaction Products. Analysis of the reaction products was made on "methionine-starved" *E. coli* sRNA that was methylated *in vitro* with [methyl-^{14}C]SAM by the enzyme fraction. After acid or alkaline hydrolysis of the methylation reaction product(s), electrophoretic resolution of the resulting nucleotides was carried out in acetic acid at pH 2.8. Identification of the resolved nucleotides was approached in two ways. (1) Chromatography of the electrophoretogram in a second dimension in isopropyl alcohol–H_2O (70:30) and ammonia in the gaseous phase to yield a map as described by Ingram and Pierce (1962). The position of each of the nucleotides was detected by its ultraviolet absorption, and

TABLE III: The Species Specificity of Rat Liver sRNA Methylases.

RNA Source	Extent of Methylation[a] (mμmoles/mg of RNA)	Pattern of Methylation (% of total radioactivity) C + A	G	U
Rat liver sRNA	0.0	–	–	–
E. coli sRNA	14.0	33	62	5
"Methionine-starved" *E. coli* sRNA	16.0	28	62	10
M. lysodeikticus sRNA	14.0	24	66	10
Yeast sRNA	1.4	43	47	10
Wheat germ sRNA	0.0	–	–	–
Calf liver sRNA	0.0	–	–	–
Chick liver sRNA	0.0	–	–	–

[a] Standard assay conditions were used (see Methods). The reaction mixture contained 0.3 μc of [methyl-^{14}C]SAM (sp act. 26.6 mc/mM, 20,000 cpm/mμmole) and 1 mg of purified enzyme fraction. The products of the reaction were hydrolyzed with 1 N $HClO_4$.

TABLE IV: Effect of Synthetic Polynucleotides on the Rate of Methylation of *E. coli* sRNA.

Polynucleotides	^{14}C Incorpd[a] (cpm)	Inhibn (%)	Pattern of Methylation (% of total radioactivity) C + A	G	U
None[b]	547	0	29	68	3
M. lysodeikticus DNA	275	50	33	64	3
Poly A	409	25	31	67	2
Poly C	426	22	33	65	2
Poly I	468	15	36	60	4
Poly U	475	13	32	65	3
Poly G	467	15	33	65	2
Poly UC (1:1), poly UA (1:1) Poly UAG (1:1:1.6) *E. coli* rRNA Rat liver sRNA	550	0	33	66	1[c]

[a] The reaction mixture contained 0.3 μc of [methyl-^{14}C]SAM (sp act. 26.6 mc/mM, 20,000 cpm/mμmole), 0.5 mg of purified enzyme fraction, 40 μg of normal *E. coli* sRNA, and 0.13 OD of polynucleotides. Incorporation in the absence of added sRNA (100 cpm) was subtracted from the figures obtained. [b] The rate of reaction without added polynucleotides was 0.135 mμmole/60 min. [c] The distribution for rat liver sRNA, the same values were obtained for other polynucleotides.

the radioactive methylnucleotides present on the chromatogram were located by cutting the paper into small strips and counting in a liquid scintillation counter. Figures 2 and 3 show typical maps of acid and alkaline hydrolysates, respectively. (2) Following electrophoresis the nucleotides were eluted from the paper and hydrolyzed with formic acid to their respective bases. The bases thus obtained were subjected to descending chromatography in isopropyl alcohol–H_2O–NH_4OH (85:15:1.3) system.

Identification of Uridylic Acid Derivatives. Using method 1 the maps obtained after acid or alkaline hydrolysis showed label in the ribothymidylic acid region (Figures 2 and 3). For method 2 the labeled RNA was hydrolyzed in 1 N $HClO_4$ and subjected to electrophoresis. The methylated base derived from the uridylic acid area was found to have the same mobility on chromatography as does thymine (Figure 4).

Identification of Guanylic Acid Derivatives. The map obtained after acid hydrolysis showed two labeled areas (Figure 2). One of them with the same mobility on chromatography as guanylic acid and a second one with a higher mobility, which may belong to N^2-

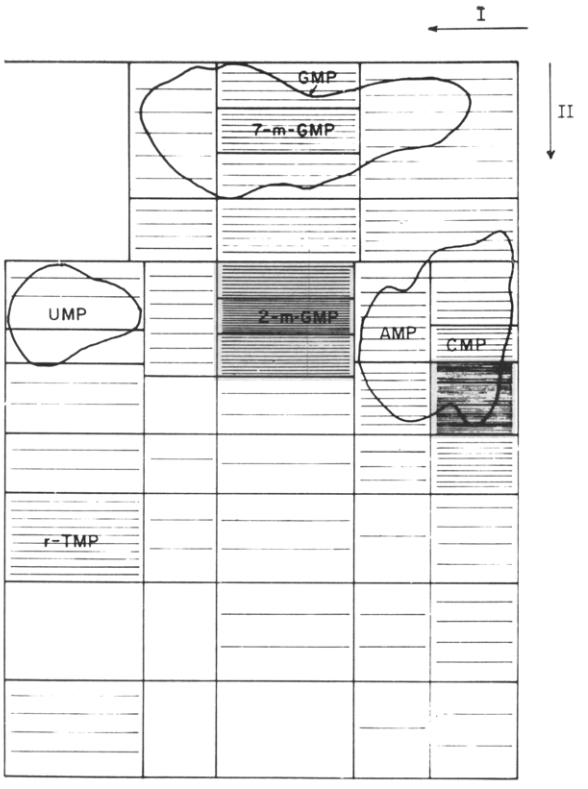

FIGURE 2: The map was prepared by: (1) electrophoresis of an acid hydrolysate of "methionine-starved" *E. coli* sRNA which was methylated with [methyl-^{14}C]SAM by the enzyme fraction, and (2) chromatography in isopropyl alcohol–water (70:30) and ammonia in gaseous-phase system in the second dimension. The paper was cut into small strips which were counted in a liquid scintillation counter. (Each line represents 20 cpm.)

methylguanylic acid. The slower moving labeled area disappeared after alkaline hydrolysis (Figure 3), suggesting that it may be the alkali-sensitive 7-methylguanylic acid. To further identify the methylated guanylic acids obtained, the labeled RNA was hydrolyzed with $HClO_4$ and subjected to electrophoresis. The methylated nucleotides were eluted from the guanylic acid area, hydrolyzed to their respective bases, and analyzed by chromatography. Two methylated bases were thus obtained with mobilities identical with 7-methylguanine and N^2-methylguanine (Figure 5). It should be noted that N^2-methylguanine is not separated in this system from N^2-dimethylguanine. Therefore, it is possible that one or both methylated bases are present in the reaction products.[2]

Identification of Adenylic Acid Derivatives. There is a difference in the electrophoretic mobility and in the chromatographic behavior of methyladenylic acid obtained after acid or alkaline hydrolysis (Figures 2 and 3). The methylnucleotide obtained after alkaline hydrolysis has the same mobility on electrophoresis as does adenylic acid and has a mobility on chromatography as expected from 6-methyladenylic acid (*cf.* Ingram and Pierce, 1962). It is known (Dunn, 1963) that 1-methyladenylic acid rearranges under alkaline conditions to form 6-methyladenylic acid. Indeed, acid hydrolysis of the RNA yielded a methylated adenylic acid that moved on the electrophoretogram somewhat slower than adenylic acid. The adenylic acid area of an acid hydrolysate electrophoretogram was eluted and hydrolyzed in formic acid, and the methylated purine derived from the eluted nucleotide was found to have the same mobility on chromatography as does 1-methyladenine and moved considerably slower than adenine or 6-methyladenine (Figure 6).

Identification of Cytidylic Acid Derivatives. Method 1 did not afford a separation between methylcytidylic acid and cytidylic acid (Figures 2 and 3). In addition, the amount of radioactivity in the cytidylic acid region, obtained after acid hydrolysis, was rather low and precluded accurate determination of 5-methylcytidylic acid by this method (Figure 3). The methylcytidylic acid was identified by using method 2. The methylated bases derived from the cytidylic acid area of the acid hydrolysate electrophoretogram were identified by chromatography as 5-methylcytosine in addition to contaminating 1-methyladenine (Figure 7). From the results presented above the following methylated bases were identified as the products of methylation of *E. coli* "methionine-starved" sRNA: thymine, 1-methyladenine, 5-methylcytosine, 7-methylguanine, and N^2-methylguanine (and/or N^2-dimethylguanine).

Comparison between Normal and Regenerating Rat Liver sRNA Methylase. In order to examine whether a correlation exists between the physiological state of rats and the activity and specificity of their sRNA methylases, we compared the enzyme from regenerating rat liver with the enzyme from normal rat liver. The specific activities and extent of methylation by crude extracts from normal and from regenerating liver, isolated 24 hr after partial hepatectomy, were compared (Table V). No significant difference between the two enzyme preparations was detected[3] nor was there any change in the pattern of label incorporated into methylated nucleotides (Table VI).

In addition the sRNAs isolated from normal liver and from regenerating liver were tested as methyl group acceptors with crude extracts from normal liver, regenerating liver, and *E. coli* cells (Table V). Whenever rat liver sRNA was used as methyl acceptor with an enzyme from rat liver, no detectable methylation occurred, regardless of the physiological state of the rat. This may mean that there are no differences in the level of methyl groups in these RNAs and no differences in the pattern of their methylated bases. Therefore, it

[2] The following chromatographic systems also failed to resolve the two bases: isopropyl alcohol–water–HCl (17:25:4.1) and *n*-butyl alcohol–ethanol–5 N HCl (3:2:2).

[3] The sRNA methylase activity of normal and regenerating liver has been recently examined independently and found to be similar (E. Borek, personal communication).

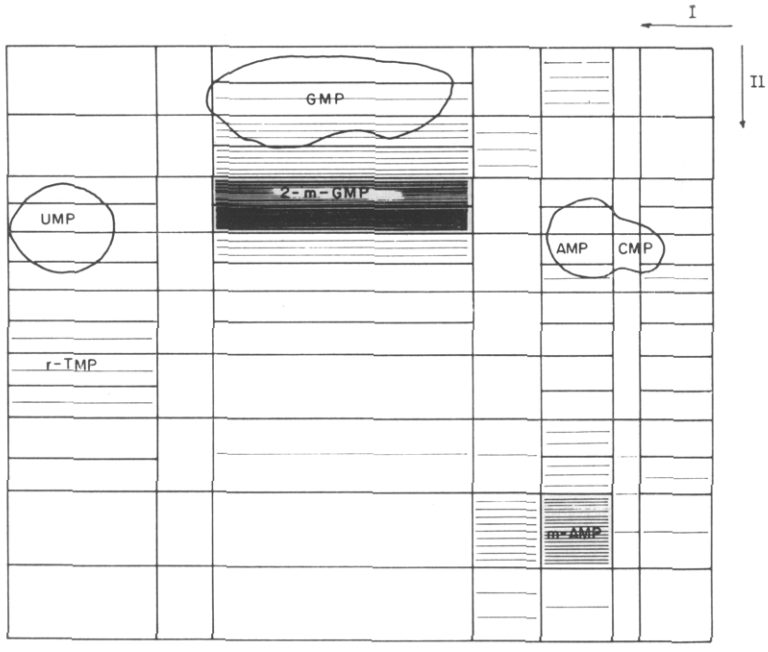

FIGURE 3: The map was prepared as described in Figure 2, but the sRNA was hydrolyzed by alkali.

seems that the specificity of the enzyme does not change. In addition, crude extract from *E. coli* methylated the two RNA preparations to the same extent, supporting the previous finding.

In order to examine further whether no differences exist between normal and regenerating rat liver sRNA, *in vivo* experiments were performed. Twenty-four hours after partial hepatectomy 0.05 mc of [methyl-^{14}C]-methionine (sp act. 29.5 mc/mM) was injected intraperitoneally into normal and hepatectomized rats. Twenty-four hours later the labeled RNA was isolated from the livers and methylated nucleotides analyzed after acid hydrolysis using method 1. The RNAs were characterized by estimating the ratio of label found in N^2-methylguanylic to that in ribothymidylic acids. We have chosen to characterize the sRNA by this ratio, since upon injection of [methyl-^{14}C]methionine into rats there is some incorporation of label into the purine and pyrimidine nucleotides, although it is very low as compared with the incorporation into the

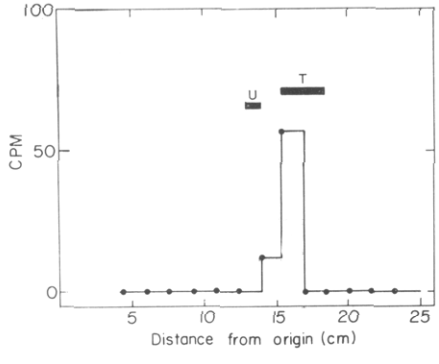

FIGURE 4: The chromatographic properties of methylated uracil and its derivatives. Uracyl and its derivatives were obtained by formic acid hydrolysis of the material eluted from the uridylic acid area derived from an electrophoretogram of acid hydrolysate of RNA which was methylated *in vitro*.

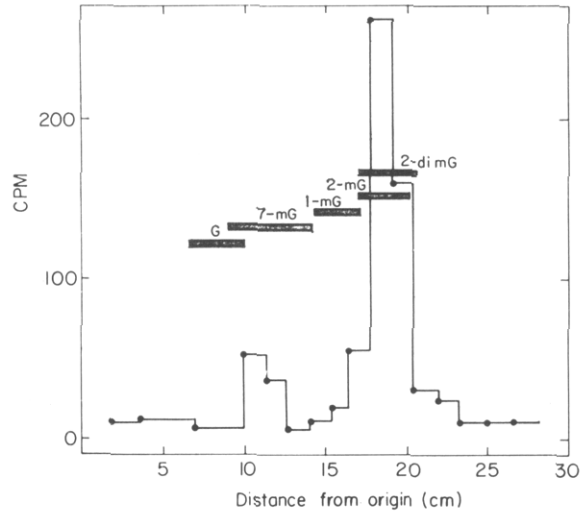

FIGURE 5: Distribution of label in chromatogram of guanine and its derivatives. Guanine and its derivatives were obtained by formic acid hydrolysis of the material eluted from the guanylic acid area derived from an electrophoretogram of acid hydrolysis of RNA which was methylated *in vitro*.

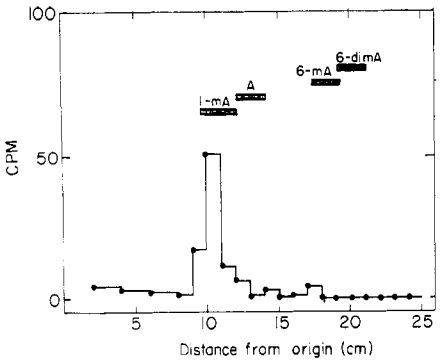

FIGURE 6: Distribution of label in chromatogram of adenine and its derivatives. Adenine and its derivatives were obtained by formic acid hydrolysis of the material eluted from the adenylic acid area derived from an electrophoretogram of acid hydrolysis of RNA which was methylated *in vitro*.

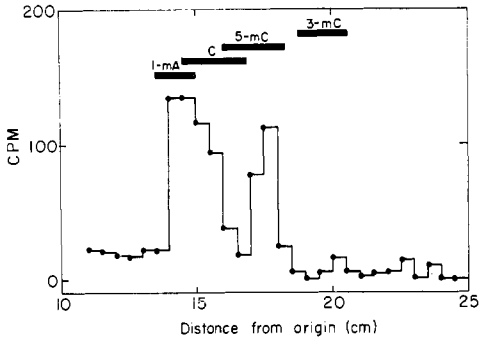

FIGURE 7: Distribution of label in chromatogram of cytidine and its derivatives. Cytidine and its derivatives were obtained by acid hydrolysis of the material eluted from the cytidylic acid area derived from an electrophoretogram of acid hydrolysis of RNA which was methylated *in vitro*.

methylated nucleotides.[4] N^2-Methylguanylic acid and ribothymidylic acid are well separated from their respective nucleotides and, therefore, are free of such contamination. As is shown in Table VII, no change in this ratio occurs using sRNA from normal and from regenerating liver. From these data it also appears that there is no difference in the pattern of methylation during regeneration. In addition it was found that the specific activities and extent of methylation by crude extract from liver of newborn rats also did not show detectable differences from those of adult rats (Table VIII).

[4] Such incorporation was not observed in *in vitro* experiments.

TABLE V: RNA Methylase Activity in Crude Extracts from Normal and Regenerating Rat Liver.

Activity	Source of Crude Extract: Regenerating Liver[a]	Normal Liver	*E. coli*[b]
Sp act. with normal *E. coli* sRNA (mμmoles/60 min mg of protein)	0.039	0.042	–
Extent of methylation of normal *E. coli* sRNA (mμmoles/mg of RNA)	12	12	–
Extent of methylation of "methionine-starved" *E. coli* sRNA (mμmoles/mg of RNA)	18	18	19
Extent of methylation of sRNA from normal liver (mμmoles/mg of RNA)	0.0	0.0	1.3
Extent of methylation of sRNA from regenerating liver (mμmoles/mg of RNA)	0.0	0.0	1.4

[a] Standard assay conditions were used (see Methods). The reaction mixture contained 0.3 μc of [methyl-^{14}C]-SAM (sp act. 29.9 mc/mM, 22,100 cpm/mμmole) and 5 μg of sRNA. [b] *E. coli* methylase activity was measured as described by Littauer and Milbauer (1965).

TABLE VI: The Pattern of Methylnucleotides in sRNA Methylated *in Vitro* by Crude Extracts from Normal and Regenerating Liver.

Source of Enzyme	Pattern of Methylation[a] (% of total radioactivity) C + A	G	U
Normal liver	61	34	5
Regenerating liver	56	38	6

[a] Analysis was carried out on methylated normal *E. coli* sRNA which was obtained as described in Table V. RNA was hydrolyzed with 1 N $HClO_4$.

Discussion

The role of methylated bases in sRNA has been studied in several laboratories and reviewed recently (Littauer *et al.*, 1966). In the case of *E. coli* phenylalanine sRNA our results (Revel and Littauer, 1966) suggest

TABLE VII: Distribution of Label in Methylated Nucleotides of Liver sRNA Derived from Rats Injected with L-[Methyl-^{14}C]methionine.[a]

Source of sRNA	^{14}C Incorporated (cpm)		
	N^2-Methylguanylic Acid	Ribothymidylic Acid	2MGP:rTP
Normal liver	320	80	4.0
Regenerating liver	947	207	4.5

[a] Normal and partial hepatectomized rats were injected (24 hr after operation) with 0.5 μc of L-[methyl-^{14}C]methionine (sp act. 29.5 mc/mM). The sRNA was isolated from the liver 24 hr later. The isolated sRNAs were hydrolyzed in 1 N $HClO_4$ and then subjected to paper electrophoresis and chromatography (see Methods), and the spots of 2MGP and rTP were counted in a liquid scintillation counter. The following abbreviations have been used in the table: 2MGP, N^2-methylguanylic acid; rTP, ribothymidylic acid.

TABLE VIII: sRNA Methylase Activity in Crude Extracts from Newborn and Adult Rat Liver.[a]

Activity	Source of Crude Extract	
	Newborn	Adult
Sp act. (mμmoles/60 min mg of protein)	0.045	0.035
Extent of methylation (mμmoles/mg of RNA)	8.0	9.0

[a] Standard assay conditions were used (see Methods). The reaction mixture contained 0.3 μc [methyl-^{14}C]-SAM (sp act. 29.9 mc/mM, 23,000 cpm/mμmole).

that methylation affects the coding properties of this sRNA; however, this need not be the only change in the function of sRNA that occurs as a result of methylation. Other possibilities have been considered for the role of methylated bases in sRNA including the involvement of methylation in some growth-regulating mechanism (Srinivasan and Borek, 1964; Littauer and Milbauer, 1965). To investigate such possible regulatory function, we have compared normal and regenerating rat liver sRNA methylases. It seemed possible that partial hepatectomy followed by liver regeneration might involve an effect on the *level* of the enzymes. However, as is seen in Table V, both normal and regenerating rat liver extracts showed the same specific activity of methylation. The results, therefore, indicate that the level of sRNA methylase(s) is the same in the two tissues. Another possibility might involve a difference in the *specificity* of the sRNA methylase(s). sRNA from regenerating liver might be supermethylated or undermethylated as compared to normal rat liver sRNA. It was observed that both sRNA preparations failed to serve as methyl acceptors with an enzyme from regenerating or normal rat liver (Table V). This observation does not necessarily mean that the two systems are homologous since it was also shown above that in some cases the rat liver sRNA methylase(s) were unable to methylate an heterologous sRNA (*e.g.*, calf liver sRNA, chick liver sRNA, and wheat germ sRNA, Table III). The possibility that such differences could be distinguished by measuring the extent or pattern of methylation of an heterologous sRNA was also tested with the two enzyme preparations. As Tables V and VI show, the extent and pattern of methylation of normal and of "methionine-starved" *E. coli* sRNA was identical whether normal or regenerating rat liver extract was used. In addition, normal and regenerating rat liver sRNA were methylated to the same extent when crude *E. coli* sRNA methylase was used (Table V).

Finally the pattern of methylation of normal and regenerating rat liver sRNA was examined after *in vivo* injection of L[methyl-^{14}C]methionine (Table VII). As expected, the *extent* of methyl group incorporation was higher in regenerating liver sRNA than in normal sRNA. This is due to the increased rate of sRNA synthesis during liver regeneration. However, the pattern of methylation did not change after hepatectomy. This result together with those discussed above are, therefore, consistent with the conclusion that the level and specificity of the two enzymes are identical.

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Characterization of Isoenzymes of Adenosine Triphosphate:D-Hexose 6-Phosphotransferase from Rat Liver*

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ABSTRACT: Four isoenzymes of adenosine triphosphate (ATP):D-hexose 6-phosphotransferase have been separated from rat liver by DEAE-cellulose. Three of these isoenzymes (A–C) are similar to animal hexokinases inasmuch as they exhibit a low K_m for glucose (10^{-5}–10^{-4} M) and the rate of phosphorylation of fructose is slightly higher than that of glucose. Isoenzyme D has been further purified by fractionation with ammonium sulfate and by chromatography on hydroxylapatite. This isoenzyme would correspond to glucokinase since it presents a high K_m for glucose (1.8×10^{-2} M), and a low activity with fructose as a substrate; it also catalyzes the phosphorylation of mannose and 2-deoxyglucose. The four isoenzymes use only ATP as phosphate donor, with K_m values of about 5×10^{-4} M. The independence of the affinity of glucokinase for glucose or ATP on the concentration of the other substrate is in agreement with the presence of two separate binding sites on the enzyme. The four isoenzymes are competitively inhibited by *N*-acetylglucosamine (K_i about 5×10^{-4} M). High concentrations of glucose inhibit isoenzymes C and D. Mannose also inhibits isoenzyme D. Glucose 6-phosphate inhibits glucokinase ($K_i = 1.5 \times 10^{-2}$ M); the inhibition is competitive with respect to ATP, while it is not clearly defined with respect to glucose.

A great deal of interest has arisen in the last few years on the regulation of glucose phosphorylating activity in rat liver. Changes in the tissue levels of ATP[1]:hexose phosphotransferase have been observed under three main circumstances: (1) variations in the supply of glucose in the diet, including total fasting (Vaughan *et al.*, 1960; DiPietro and Weinhouse, 1960; Niemeyer *et al.*, 1962, 1963; Pérez *et al.*, 1964); (2) availability of insulin (DiPietro and Weinhouse, 1960; Salas *et al.*, 1963; Niemeyer *et al.*, 1967); and (3) initial stages of development after birth (Walker, 1963). Of special significance were the reports by Walker (1963) and Viñuela *et al.* (1963), describing two protein fractions with glucose phosphorylating activity in liver. Only one of these proteins, characterized by its high K_m for glucose and called glucokinase, was amenable to changes under the various above-mentioned conditions. We separated four fractions or isoenzymes[2]

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[1] Abbreviations used: ATP, adenosine triphosphate; $NADPH_2$, reduced nicotinamide–adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate; NAD^+, oxidized nicotinamide–adenine dinucleotide; $NADH_2$, reduced nicotinamide–adenine dinucleotide; UTP, uridine triphosphate; Glc-6-P, glucose 6-phosphate.

[2] The term isoenzyme is used here in the broad sense accepted by the Standing Committee on Enzymes of the International Union of Biochemistry (Webb, 1964).