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*A NEW ENZYME OF RNA SYNTHESIS: RNA METHYLASE**

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Soluble RNA is characterized by the presence in small amounts of a variety of methylated purines and pyrimidines. The occurrence of these minor components appears to be universal: the relative amounts, however, are variable. It has been recently demonstrated in our laboratory that the methyl groups of all of these bases in bacterial RNA originate from methionine via the same methyl pool.[1] Biswas, Edmonds, and Abrams[2] have extended these observations to ascites cells and showed methionine to be the methyl donor for the methylated guanines in those cells as well.

The synthesis of 5-methyl uracil (thymine) of RNA in this manner is particularly significant, since the same base intended for incorporation into DNA is produced by an entirely different pathway, from deoxyuridine monophosphate and N^5, N^{10}-methylene tetrahydrofolate.[3]

It was observed several years ago that the methionine auxotroph *E. coli* $K_{12}W6$, unlike other amino acid auxotrophs known at that time, continues the synthesis of RNA during starvation of its essential amino acid.[4] The accumulation is not unique for methionine, for Stent and Brenner have recently shown that double amino acid auxotrophs derived from *E. coli* $K_{12}W6$ accumulate RNA almost as well on deprivation of other amino acids.[5]

Analysis of the RNA accumulating on methionine starvation revealed that even though most of the RNA has sedimentation characteristics of the soluble species,[6] the methylated minor components were lacking from it.[7] The origin of all the methyl groups of the soluble RNA from the same methyl pool and the observed synthesis of soluble RNA lacking the methylated bases on methionine starvation suggested to us the possibility that methylation occurs not at the level of some monomer but at the level of the polynucleotide.[8] According to this view, the accumulation of nonmethylated RNA must be the sequential result of two separate deficiencies: first, the lack of control and continued synthesis of RNA in the absence of an amino acid, and, second, the failure of methylation of the accumulating RNA due to the absence of the methyl source.

We wish to report here evidence from experiments both *in vivo* and with cell-free extracts for the existence of an enzyme which can achieve methylation of S-RNA at the polynucleotide level.

In Vivo Experiments.—In these experiments, *E. coli* $K_{12}W6$ were allowed to accumulate RNA as a result of methionine starvation.[4] The cells enriched with RNA were removed from the starvation medium, washed, and incubated in fresh

complete culture medium containing $P^{32}O_4$ and methionine-methyl-C^{14} in a known ratio. At 15-min intervals, samples of the culture were removed, and RNA was isolated by the method of Littlefield and Dunn[9] and was dialyzed against $PO_4^{\equiv}$ buffer to remove $P^{32}O_4^{\equiv}$. The C^{14}/P^{32} in the RNA was determined by counting in a liquid scintillation counter. In parallel experiments, organisms in logarithmic growth phase were deprived of methionine for 15 minutes to deplete their internal pool and were exposed to a complete culture medium containing the same C^{14}/P^{32} ratio which was used for the starved organisms. RNA was isolated as before, and the C^{14}/P^{32} ratio was determined to establish the ratio of new nucleotide bonds formed per methyl group introduced into the RNA of normal organisms. In Figure 1, the results of these experiments are summarized.

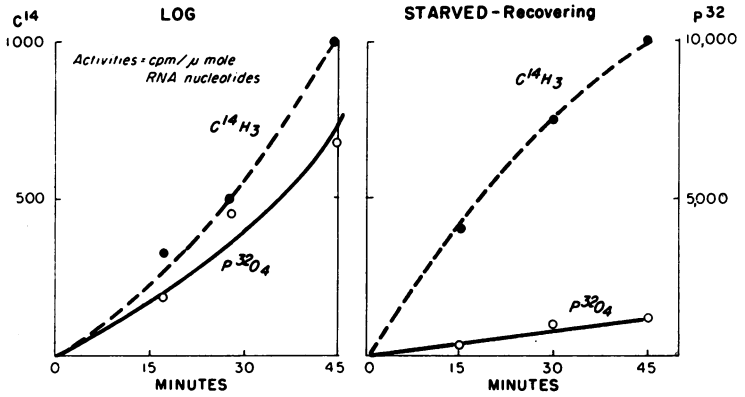

FIG. 1.—The incorporation of $C^{14}H_3$ and $P^{32}O_4$ into the RNA of organisms in logarithmic growth phase (left panel) and of organisms which had been pre-starved of methionine (right panel). For details see text.

It can be calculated that in the organisms containing the preformed, nonmethylated RNA, the acquisition of a methyl group per new nucleotide bond is greater by a factor of 8 during the first 15 min than in a culture in logarithmic growth phase.

Ribosyl thymine 3′-phosphate labeled both with C^{14} and P^{32} was isolated from the RNA samples of the experiments described above, and the C^{14}/P^{32} ratios were determined in the nucleotides.‡ The data obtained are presented in Table 1. During the first 15 min of incubation in the organisms with the accumulated RNA, methylation was in excess of nucleotide bond formation by a factor of 200 over that occurring in the organisms in logarithmic growth phase.

TABLE 1

P^{32} TO C^{14} RATIOS IN RIBOSYL THYMINE 3′PHOSPHATE FROM STARVED AND NORMAL CELLS
(P^{32} Activity Cpm found per 10^4 Cpm of C^{14} H_3)

Time of incubation	Normal cells	Pre-starved cells
15′	600	3
30′	800	30

Our interpretation that these data represent methylation of uracil at a polynucleotide level in the prestarved organisms would be refuted if there were extensive depolymerization of the accumulated RNA to the nucleotide level within the recovering cells followed by resynthesis of the newly methylated nucleotides. Such a pathway could shun the labeled $P^{32}O_4^{\equiv}$ of the medium. However, we could de-

tect no depolymerization of the accumulated RNA. Moreover, it has been found by Kahan and Hurwitz[11] that *E. coli* lack the kinase for the synthesis of ribosyl thymine 5′ triphosphate. Therefore, since this precursor's synthesis cannot be achieved by *E. coli*, such a pathway is apparently impossible.

But, for the unequivocal demonstration of methylation of a pre-formed polymer, a study of such a mechanism was undertaken in a cell-free system.

Experiments with Cell-Free Systems.—The substrate for these methylation studies was the s-RNA accumulated during methionine starvation. For comparison, similar preparations were made from organisms in logarithmic growth phase. About 10^{12} cells which had been starved of methionine for 3 hr were washed with isotonic KCl and were ground, while cold, with twice their weight of alumina. The paste was extracted with 0.01 *M* Tris buffer at pH 7.8 containing 10^{-4} *M* $MgCl_2$ and 5 μg DNAase per ml. The extract was clarified by centrifugation for 20 min at 20,000 × *g* and then centrifuged at 100,000 × *g* for 3 hr. To the clear supernatant fluid an equal volume of water-saturated phenol was added and the mixture was shaken at room temperature for 15 min. The RNA was precipitated from the aqueous layer with two volumes of cold ethyl alcohol. The precipitated RNA was dissolved in a small volume of water and was dialyzed against 10^{-3} *M* Tris buffer at pH 7.8. The solution of RNA was lyophilized and stored in the cold. The two RNA preparations—the nonmethylated and methylated—were used in solutions at a concentration of 10 mg/ml. The sedimentation character-

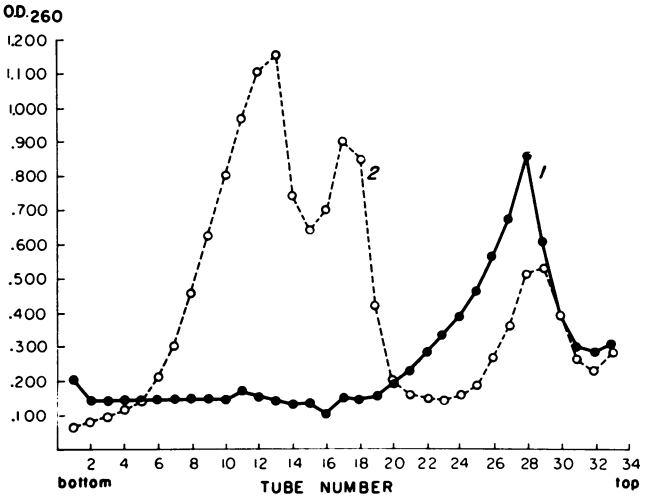

FIG. 2.—Curve 1 is the sedimentation pattern in 5–20% sucrose gradient centrifugation of the s-RNA used as substrate for methylation. Curve 2 represents the sedimentation pattern, under similar conditions, of the total RNA of organisms in logarithmic growth phase. The duration of the centrifugation was 12 hr in a swinging bucket rotor at 53,500 × *g*.

istics of the nonmethylated s-RNA in a 5–20 per cent sucrose gradient centrifugation are shown in Figure 2.

The soluble enzyme was prepared from organisms in logarithmic growth phase. About 10^{12} cells were washed with isotonic KCl and were ground with two times their weight of alumina. The paste was extracted with 10 ml of a buffer solution

at pH 8 which contained the following ingredients: 0.01 *M* Tris, 0.1 *M* $MgCl_2$, 0.015 *M* GSH, and 5 μg DNAase per ml. The mixture was clarified by centrifugation for 20 min at 20,000 $\times$ *g* and was then centrifuged for 2 hr at 100,000 $\times$ *g*. The supernatant solution was stored frozen in small lots.

The incubation mixture for the methylation studies contained 16 μM phosphoenol pyruvate, 20 μg phosphoenol pyruvate kinase, 2 μM ATP, 0.2 μM C^{14} (methyl) methionine, 2 ml enzyme extract, 2 mg RNA preparation, and pH 7.8 0.01 *M* Tris buffer to yield a total volume of 5.0 ml. The incubation was performed at 30° for 30 min, at the end of which time the pH of the solution was raised to 9.5–10 with 2 *M* Na_2CO_3 and it was incubated at 30° for 1 hr, to dissociate the methionine from the RNA. The RNA was re-isolated by precipitation with 10 per cent TCA.

The ribosyl thymine 3′phosphate was isolated as before and its specific radioactivity was determined. In Table 2, some of the results of these methylation studies—as expressed by the synthesis of C^{14} thymine—are presented. While in this communication we present evidence only for the methylation of uracil, autoradiography revealed the methylation of other bases as well in the same cell-free system.

TABLE 2

$C^{14}H_3$ INCORPORATED INTO RIBOSYL THYMINE 3′PHOSPHATE IN A CELL-FREE SYSTEM

(cpm found per 10^{-2} μM of the nucleotide)

Addition	cpm
0	6
2 mg s-RNA from normal cells	5
2 mg s-RNA from starved cells	750

For other components of the incubation mixture see text.

It is apparent from these data that a soluble enzyme, RNA methylase, exists in *E. coli* which can methylate the base components of soluble RNA when the latter are already in some polynucleotide form. A study of the precise nature of the receptor for the methylation as well as the purification of the enzyme are in progress.

The operation of a methylating enzyme at the level of polynucleotides is a second example of the alteration of a nucleic acid whose primary structure is previously determined during its assembly. Kornberg, Zimmerman, and Kornberg[12] have shown that glucosylation of 5-hydroxymethyl cytosine in the DNA of the T-even phages occurs at the level of the polymer.

Methylation of RNA may alter the secondary or tertiary structure of the polynucleotide or it may serve to reduce the number of sites of hydrogen bonding. The latter may be an asset in transfer RNA whose function is currently visualized as involving the making of transient, reversible attachments to some binding sites.

On the other hand, it is also possible that methylation merely serves to reduce the susceptibility of transfer RNA to hydrolysis by the nucleases which abound in the ribosomes. This might help to conserve a molecular species whose structure is constant and whose function is repetitive.

Transfer RNA is thought to have complete specificity for each amino acid. How the sites of specificity are retained during methylation is obscure and clarification awaits the determination of the pattern of methylation.

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*STRUCTURE OF FRAGMENTS OF ANTIBODY MOLECULES AS REVEALED BY REDUCTION OF EXPOSED DISULFIDE BONDS**

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Antibody molecules seem to be formed from two fragments, each containing one specific combining region, and a third fragment which does not contain such a binding site.[1–5]

We have now found that the univalent fragments in the case of several antibody preparations appear to be composed of more than one polypeptide chain held together by disulfide linkages and that these disulfide linkages are susceptible to reduction without prior denaturation of the fragment.

The method used stems from the reports[6, 7] by Edelman and co-workers (see also Franek,[8] and Ramel *et al.*[9]) that γ-globulin and antibody molecules are dissociated into smaller units when reduced in the presence of 8 *M* urea and from the observation[10] in our laboratory that two of the disulfide bonds in fragments of γ-globulin derived by papain digestion are easily reducible, while the others are made available for reduction only in 8 *M* urea.

The work reported here shows that when a fragment of rabbit antibody is reduced in the absence of urea there results an altered fragment with a still intact combining region. This reduced fragment upon exposure to urea falls apart into two or more polypeptide chains.

Materials and Methods.—Antisera: Pooled rabbit antihapten antisera were obtained from groups of 5 to 12 animals. The antigens were prepared by coupling the following substances to bovine γ-globulin (BGG): diazotized *p*-aminobenzoate (to give BGG-X_p), diazotized *p*-aminobenzenearsonate (BGG-R_p), and diazotized