THE INCORPORATION OF METHYL GROUPS INTO AMINO ACID TRANSFER RIBONUCLEIC ACID*

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The presence of methylated purine and pyrimidine bases in the amino acid transfer or soluble RNA (S-RNA) of both mammalian and bacterial cells has been known for several years (Dunn, 1959). The methylated bases, which occur in different relative concentrations in each species (Smith and Dunn, 1959), are not incorporated as such (Starr, 1962) but apparently are formed by methylation of the preformed polynucleotide chain (Fleissner and Borek, 1962). Methionine acts as the methyl donor in the intact cell (Biswas et al, 1961; Mandel and Borek, 1961) as well as in the in vitro cell-free preparations (Fleissner and Borek, 1962).

The function of the methyl groups is not known. Fleissner and Borek (1962) have suggested that the methylation alters the secondary and tertiary structures of the polynucleotides, or reduces the number of sites of hydrogen bonding, thereby facilitating transient attachment to binding sites. The studies reported in this and the following paper were undertaken in order to confirm the presence of the methyl groups in the soluble RNA fraction and to determine whether the pattern of methylation is involved in the determination of amino acid specificity of S-RNA.

METHODS

The "relaxed" methionine-requiring E.coli Mutant K12W6 was grown in a salts-glucose medium with added methionine, 20 μgm/ml. The cells were harvested in the logarithmic growth phase, washed twice with saline solution, and resuspended in

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methionine-free medium. The bacteria were reincubated at 37° for one hour beyond the time at which endogenous methionine was depleted and growth, measured by O.D. at 660 mμ, stopped. S-RNA was extracted from the methionine-starved bacteria by a modification of the direct phenol extraction method of von Ehrenstein and Lipman (1961) or by phenol extraction of the 105,000 xG supernatant. The amount of S-RNA synthesized per gram wet weight of the bacteria was measured by determining total O.D. at 260 mμ. The degree of methylation of S-RNA was determined by measuring specific activity of S-RNA extracted from E.coli K12W6 which had been grown prior to starvation in the presence of methyl-C^{14}-methionine. In the in vivo methyl incorporation studies, E.coli K12W6 was grown in the presence of methionine, 20 μgm/ml. In mid-logarithmic growth phase, methyl-C^{14}-methionine was added to the incubation medium to produce a specific activity of 1.8×10^5 CPM/μmole methionine. Incubation was continued 15 more minutes prior to harvesting. Total cellular RNA was collected by grinding the cells with 3 times their weight of alumina in the presence of 0.1 volume/wt. of 2% Duponol, followed by extraction with 3 volumes of 0.05M Tris buffer pH 7.4 containing 0.005M MgAcetate and 0.2% Duponol. Cell debris and alumina were removed by centrifugation at 30,000 xG for one hour. The RNA was obtained from the resulting supernatant solution by phenol extraction, and was purified by a second phenol extraction and incubation in 0.5M Tris buffer, pH 8.8, to remove amino acids which had been incorporated into S-RNA. The RNA was precipitated by the addition of 2 volumes of alcohol and was redissolved in 0.005M Tris, pH 7.4 containing 0.0001M MgAcetate.

The enzyme RNA methylase was extracted from normal E.coli and assayed by methods similar to those reported by Fleissner and Borek (1962).

The sedimentation characteristics of the various RNA samples were determined by centrifugation in a 5 to 20% sucrose gradient containing 0.005M Tris, pH 7.5, and 0.0001M MgAcetate. Two drop samples were collected and analyzed for both ultraviolet adsorption at 260 mμ and for radioactivity. The latter was measured in a Tri-Carb liquid scintillation spectrometer.

Incorporation of C^{14}-methyl into "Methyl-Poor" RNA: The in vitro incorporation of C^{14}-methyl groups into S-RNA isolated from normal E.coli B and methionine-starved

E.coli K12W6 is reported in Table II. It may be noted that only the RNA prepared from the starved mutant, which was shown to contain less than half the normal amount of methyl groups, is capable of being significantly methylated by RNA methylase. Under the conditions of the incubation, a maximum of 10 mμmoles CH_3/mgm RNA are incorporated, although the RNA is deficient by approximately 30-40 mμmoles/mgm (Table I) Normal RNA, in contrast to the "methyl-poor" RNA, is apparently nearly saturated with respect to sites which may be methylated. These results confirm those reported by Fleissner and Borek (1962).

Table I

Effect of Methionine Starvation on Synthesis and Methylation of S-RNA

	Normal Mid-Log Phase	Normal Plateau Phase	Methionine Starvation
mgm. S-RNA per gm. wet weight of bacteria	2.40	2.45	4.60
mμmole $C^{14}_{*}H_3$ per mgm. S-RNA	56	61	21

* Based on the assumption that all C^{14} present in the S-RNA exists as CH_3 rather than as portions of the bases incorporated via formate derivatives.

Table II

Incorporation of C^{14}-Methyl Groups into Normal and Methyl-Poor S-RNA

Incub. Time (min.)	mμmole CH_3 incorporated/mgm S-RNA	
	Normal S-RNA	CH_3-Poor S-RNA
15	0.2	3.5
30	0.7	5.8
60	1.0	9.0

Incubation mixture included crude E.coli extract 0.25 ml, RNA 1.0 mgm, $MgCl_2$ 5.0 μmoles, PEP 5.0 μmoles, Pyruvic Kinase 15 μgm, K_2HPO_4 buffer, pH 7.4, 50 μmoles, ATP 2.0 μmoles, $C^{14}H_3$-methionine 5.0 μmole (126×10^3 CPM/μmole, in a volume of 1.0 ml. Incubation was at 37°.

Sedimentation Analysis of RNA Methylated in vitro: In order to confirm the assumption that the methyl groups are incorporated into S-RNA, the "methyl-poor" RNA C^{14}-methylated *in vitro* by the RNA methylase was reisolated by phenol extraction. Methionine incorporated into the terminal position of S-RNA as the whole amino acid was removed by incubation in 0.5M Tris buffer, pH 8.8, at 37°C for 45 minutes. The RNA was purified as described in Methods. The sedimentation characteristics of this RNA were analyzed in the same system used for whole cell RNA. The results are shown in Figure II. It can be seen that the homogenous 4S peak of S-RNA corresponds exactly with the distribution of radioactivity.

DISCUSSION

The results of the experiments described are consistent with the assumption that methylated bases present in trace amounts in S-RNA are indeed native to this fraction rather than to another species of RNA of higher molecular weight but isolated fortuitously with S-RNA as a consequence of the method of preparation. Although the absence of C^{14}-CH_3 in the 8-16S region would indicate that degraded messenger RNA is not the source of methylated bases found in the S-RNA fraction, experiments in which messenger RNA can be identified are being carried out to confirm this. Ribosomal RNA, even should it prove to contain methylated bases, could not be the source of the bases which are found in S-RNA.

RESULTS

Synthesis and methylation of S-RNA during methionine starvation: Borek (1955) has reported the doubling of total cellular RNA during methionine-starvation and has given indirect evidence for synthesis of messenger RNA (Mandel and Borek, 1962). In the present experiment, the amount of S-RNA per gram wet weight of cells was shown to approximately double (Table I). The number of C^{14}-methyl groups per mgm S-RNA decreased by more than half, confirming not only continued synthesis of unmethylated S-RNA in the absence of methionine, but also suggesting some demethylation of preformed RNA (Table I). Identification of the individual methylated bases was not attempted. The number of methyl groups per total nucleotides in normal S-RNA, 2%, is in the range reported by Dunn et al, (1960).

Site of C^{14}-methyl incorporation in vivo: This experiment was carried out in order to determine whether the methylated bases were actually in S-RNA or were in an RNA species of higher molecular weight which might have been isolated along with the soluble fraction. This possibility was suggested by the fact that any one of the methylated bases constitutes less than 1% of the total bases of the S-RNA. Since S-RNA molecules consist of only 100 bases or less, these trace bases could not be distributed evenly among all of the RNA molecules. In Figure I, however, C^{14}-methyl

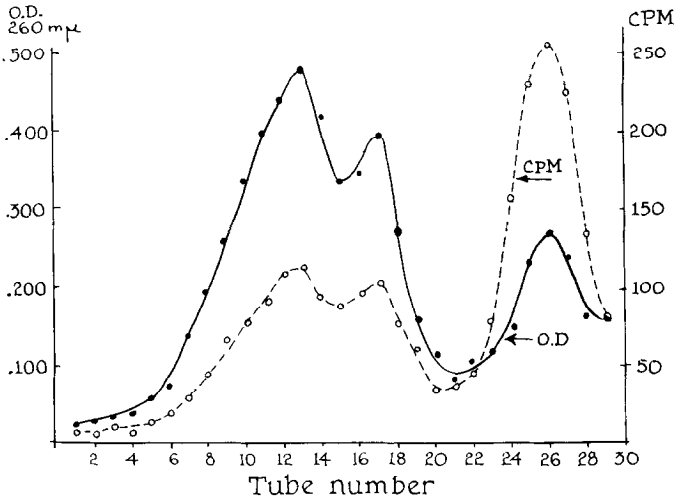

Figure I. Sedimentation pattern of whole cell RNA C^{14}-methyl-labelled in vivo. Centrifugation was for 4 hours at 37,000 RPM in Spinco SW39 rotor.

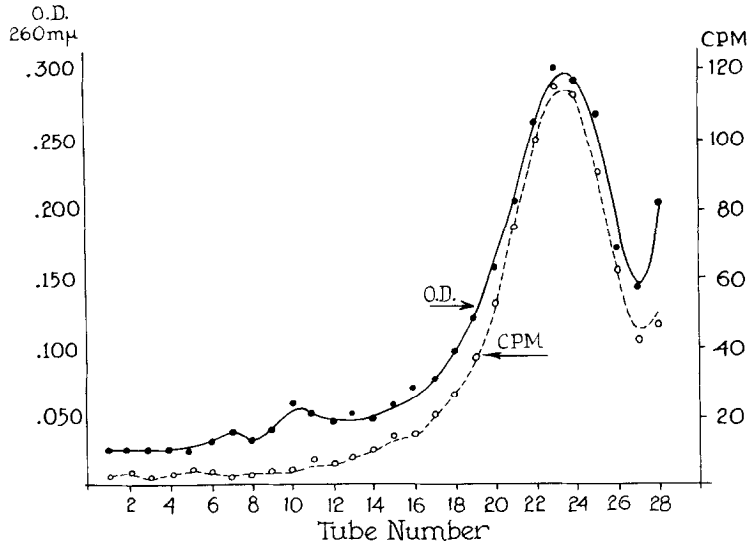

Figure II. Sedimentation pattern of methyl-poor S-RNA C^{14}-methyl-labelled in vitro. Conditions as in Figure I.

groups are shown to occur in the greatest relative concentration in the 4S fraction of total cellular RNA. Radioactivity probably due to the presence of methyl groups was consistently found in association with ribosomal RNA, even after double phenol extraction and treatment with weak alkali. We are currently studying the source of this radioactivity, which could be due to 1) binding of S-RNA to ribosomal RNA (Smith, 1962); 2) presence of methylated bases in ribosomal RNA in amounts previously thought to be due to contaminating S-RNA (Bergquist and Matthews, 1962); or 3) contribution of small amounts of C^{14}-CH_3 to the 1-carbon pool of purine and pyrimidine precursors (MacKenzie and duVigneaud, 1950). In any event, methylated bases in stable ribosomal RNA could not be the source of the methylated bases found in the soluble fraction. Significant radioactivity was not observed in the 8-16S region.

Since normal RNA is incapable of being methylated under the conditions of assay of RNA methylase, it would appear that the methyl groups are not incorporated randomly along the molecule but enter only at specific sites. At the present time, the factors which determine the specificity of methylation remain to be elucidated.

The following paper describes studies on the incorporation of amino acids into "methyl-poor" S-RNA.

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