# EVIDENCE FOR ENZYMATIC METHYLATION IN VITRO OF THE RIBOSE MOIETY OF RNA

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Since Smith and Dunn (1959) isolated 2'-O-methyladenosine from wheat germ and rat liver tRNA, five types of 2'-O-ribose methylated nucleosides have been found in tRNA from different sources (Hall, 1964). The distribution of the 2'-O-methylnucleosides in tRNA is nonrandom and differs from one species to another (Gray and Lane, 1967), which indicates some specificity in the biosynthesis of these compounds. The mechanism of biosynthesis of the 2'-O-methylnucleosides has so far not been elucidated. Dubin and Günalp (1967) and Phillips and Kjellin-Stråby (1967) have some evidence for that *in vivo* the methyl groups in ribose originate from methionine. In this paper we describe a reaction *in vitro* with an enzyme preparation from *Saccharomyces cerevisiae*, indicating that a methylation of the ribose moiety in the 2'-O-position occurs on the polynucleotide level. The *in vitro* methylated tRNA from

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Abbreviations used: N, nucleoside, $N_x$, nucleoside containing a methyl group in the 2'-O-position of the ribose, $N_xpN$, alkali-stable dinucleoside monophosphate, $N_xpNp$, alkali-stable dinucleotide, Ar, adenosine.

Escherichia coli was hydrolyzed in alkali and treated with phosphatase. The nucleosides and dinucleoside phosphates formed were separated by paper electrophoresis. The isolated dinucleoside phosphates were degraded enzymatically to nucleosides and analyzed by paper chromatography. One of the 2'-O-methylnucleosides formed has been preliminarily identified as 2'-O-methyladenosine.

RESULTS

Transfer RNA was prepared as described by Svensson (1967) from E. coli W6, grown with a supply of methionine (Svensson et al., 1963). The preparation of a protein fraction, containing tRNA-methylating enzymes, from the yeast strain S. cerevisiae D84 (Phillips and Kjellin-Stråby, 1967) will be described in detail elsewhere. The essentials of the procedure are pressing of cells, treatment with DNase, centrifugation, ammonium sulfate precipitation and dialysis.

The incubation mixture for methylation of tRNA in vitro contained in a total volume of 2 ml: 100 mM Tris-HCl, pH 8.0, 2 mM glutathione, 10 mM $MgCl_2$, 20 mM $NH_4Cl$, 0.1 mM EDTA, 41 mg of protein, 8 mg of tRNA and 4.9 $\mu$M of [$^{14}$C-methyl] S-adenosyl-L-methionine (50.5 mC per mmole). The mixture was incubated at 30° for 7 hours, and the reaction was stopped by shaking with phenol. The tRNA was precipitated twice with ethanol and hydrolyzed in 0.2 M NaOH at 37° for 16 hours, resulting in dinucleotides of the type $N_xpNp$ in addition to 3'-nucleotides, nucleosides and nucleoside diphosphates. The alkaline hydrolysate was neutralized with Amberlite IRC-50 ($H^+$). The ion exchanger was repeatedly washed with water, and the combined solutions were taken

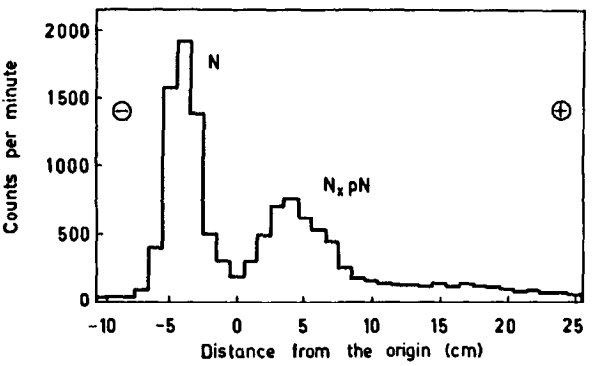


Fig. 1. Electrophoretogram of nucleosides and dinucleoside monophophates.

to dryness in a stream of air. The residue was dissolved in 500 µl of 0.1 M triethylammonium bicarbonate - 1 mM $MgCl_2$, pH 9.0, and incubated at 37° for 6 hours with 0.15 mg of alkaline phosphatase. The solution, now containing nucleosides and dinucleoside monophosphates of the type $N_xpN$, was chromatographed on Whatman 3 MM paper in 1-butanol-water (86:14), ammonia (gas) at 24° for 25 hours. In this way most of the nucleosides are separated away from the almost stationary $N_xpN$. A region from 0.5 cm behind and up to 2.0 cm in front of the origin was eluted and subjected to electrophoresis according to Markham (1965) in 0.01 M potassium phosphate buffer, pH 7.2 (40 V/cm; 90 min).

The dinucleoside phosphates migrate towards the anode according to the marker thymidylyl-thymidine and are separated from the nucleosides which slowly migrate towards the cathode (Fig. 1). The dinucleoside phosphate region was eluted and the solution was taken to dryness. The residue was dissolved in 125 µl of 0.1 M triethylammonium bicar-

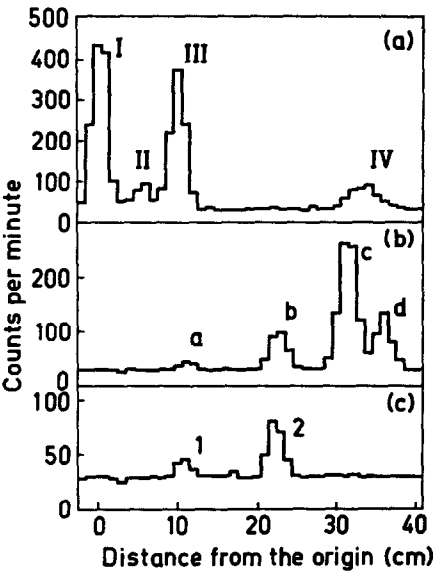


Fig. 2. Chromatographic fractionation of some nucleosides obtained from alkali-stable dinucleoside monophosphates. (a) Solvent system A: ethyl acetate-1-propanol-water (4:1:2); the top phase was used. (b) Peak IV fractionated in solvent system B: 2-propanol-conc. hydrochloric acid-water (680:170:144). (c) Peak IV b fractionated in 1-butanol-water-conc. ammonia (86:14:5). The chromatograms were developed at 24° for (a) 15 hours, (b) 25 hours, and (c) 15 hours.

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bonate - 1 mM $MgCl_2$, pH 9.0, and incubated for 21 hours at 37° with a mixture of 0.025 mg of E. coli alkaline phos-

TABLE I

Chromatographic comparison of product with authentic markers. Solvent systems A and B are described in the legend to Fig.2. System C is 1-butanol-water (86:14), ammonia (gas). Figures are expressed as $R_{Ar}$-values.

| Solvent system | A | B | C |
|---|---|---|---|
| 2'-O-methylguanosine | 0.73 | 1.50 | 0.46 |
| 2'-O-methyladenosine | 2.06 | 1.57 | 1.74 |
| Peak IV b 2 | 2.10 | 1.60 | 1.74 |

phatase and 0.05 $A_{280}$-units of snake-venom phosphodiesterase from the first step of the preparation procedure described by Björk (1963). The resulting radioactive nucleosides were fractionated by paper chromatography in three different systems, resulting in a variety of peaks. Part of this fractionation is shown in Fig. 2. One of the isolated peaks (IV b 2) was characterized by rechromatography in three systems in the presence of markers for 2'-O-methyladenosine and 2'-O-methylguanosine. Table I shows that the material in this peak had the same $R_{Ar}$-values as 2'-O-methyladenosine in all three systems. The labelled material of peak IV b 2 was also shown to be resistant to periodate treatment as expected for nucleosides methylated in the 2'-O-position.

To exclude the possibility of nonenzymatic methylation of the ribose moiety in tRNA, a control experiment was performed. E. coli tRNA was incubated as described above, except for omission of enzyme, and treated according to the procedure outlined. No radioactivity was found in the

TABLE II

Recovery of radioactivity in methylated tRNA and in fractionated hydrolyzation products. All values are given in Cpm, corrected for background radiation.

| Incorporation into | Reaction mixture | | |
|---|---|---|---|
| | Complete | - Enzyme | - tRNA |
| tRNA (0.1 g) | 250,000 | 860 | - |
| Nucleosides after electrophoresis | 36,500 | 180 | 750 |
| $N_xpN$ after electrophoresis | 18,260 | 80 | 150 |
| Peak IV | 1,290 | 0 | 0 |
| Peak IV b | 170 | - | - |
| Peak IV b 2 | 120 | - | - |

region where peak IV (see Fig. 2a) was expected (Table II). A control experiment was also made omitting E. coli tRNA from the reaction mixture. Table II shows that the 2'-O-methyladenosine formed does not originate from RNA present in the yeast enzyme preparation but from E. coli tRNA.

## DISCUSSION

Our results suggest that, in analogy with the biosynthesis of methylated bases in RNA, the 2'-O-methylated nucleosides are enzymatically synthesized on the polynucleotide level. One of the methylated products formed by the yeast enzyme in the presence of E. coli tRNA has been characterized as 2'-O-methyladenosine. The low yield of 2'-O-methyladenosine may reflect that E. coli tRNA is a poor substrate for heterologous ribose methylase or that the reaction conditions are not appropriate.

A full report will be published elsewhere.

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