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Methylation of t-RNA: Effect of Vitamin B_{12} Deficiency in Rat. (31701)

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Dunn *et al*(1) first reported the presence of small amounts of methylated purines and pyrimidines in amino acid transfer ribonucleic acid in addition to the 4 main bases. The methyl groups of these bases were shown to be derived by methionine by transmethylation at the polynucleotide level of the previously formed t-RNA(2,3). Evidence for this was first obtained by Borek and his associates(4) by examination of the RNA that accumulated during methionine starvation of the auxotroph of *Escherichia coli* K_{12} W_6 which requires the essential amino acid. The newly formed t-RNA lacked the methylated bases which pointed to the possibility that methionine in the form of S-adenosylmethionine was the active methyl donor. This was later confirmed by both *in vitro* and *in vivo* systems using labeled methionine(5,6). Following these findings, attempts were made to study the enzymatic methylation of methyl-deficient t-RNA in cell-free systems using enzymes which are called "RNA methylases". Hurwitz and his group(7) were mainly responsible for isolating several of these enzymes which were shown to be specific for the individual bases. These enzymes were shown to be capable of further methylating an otherwise fully methylated t-RNA from a different organism(8,9). Trace amounts of methylated bases are also found to occur in ribosomal RNA(10) and enzymes which methylate ribosomal RNA have been discovered recently (11,12,13). The purpose of our present study was to examine the possibility of an involvement of vitamin B_{12} in a pathway of methylation of ribonucleic acids in rat liver. Preliminary results presented here lead us to the conclusion that a vitamin B_{12} requiring pathway of t-RNA methylation does exist.

Materials and methods. Young male rats (45-50 g) were divided into 3 groups and were fed a soya-lactose basal diet(14) devoid of vit. B_{12} and methionine. Group II received, in addition, cyanocobalamin (50 μg/kg diet) and Group III methionine (2 g/kg diet). The animals were maintained on these diets for a period of 8 weeks and were then sacrificed by decapitation after fasting overnight to deplete their liver glycogen. The livers were homogenized in 5 volumes of 0.25 M sucrose and the cell supernatant fractions were obtained by centrifugation at 105,000 $\times$ *g* for one hour in a Spinco Model L centrifuge after a preliminary centrifugation at 10,000 $\times$ *g*. A small portion of the supernatant was used as the source of the methylating enzyme while a major portion of it was utilized for the isolation of t-RNA by the phenol extraction procedure of Kirby(15). The isolated t-RNA was purified by dialysis against several volumes of tris buffer (1×10^{-3} M and pH 7.8). The purity of isolated t-RNA was assessed by its absorption spectrum in the U-V region. It showed a single peak at 260 mμ and the absorption minimum was at 232 mμ. The ratios of A_{280}/A_{260} and A_{232}/A_{260} were 0.53 and 0.52 respectively.

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TABLE I. *In vivo* Incorporation of $C^{14}H_3$-Methionine and Serine-3-C^{14} into Rat Liver t-RNA.

C^{14}-compound	Liver t-RNA total radioactivity (cpm) Basal	Basal + B_{12}	Basal + methionine
$C^{14}H_3$-methionine	5522	4473	4168
	5472	4644	4128
Avg	5497	4559	4148
Serine-3-C^{14}	3937	3885	1774
	3960	3630	1800
Avg	3949	3758	1787

Animals were injected intraperitoneally with a single dose of radioactive compound (10 μc/100 g body wt) and were sacrificed 6 hr later. T-RNA was isolated from the 105,000 *g* supernatant of the liver homogenates and was counted as described in text.

The methylating enzyme was partially purified from the cell supernatant by ammonium sulfate precipitation (40-50% saturation) following an initial treatment with streptomycin sulfate and centrifugation to remove the precipitate. The ammonium sulfate-precipitated enzyme was dissolved in a small volume of water and was dialysed against 1 m M tris-HCI buffer overnight in cold. In *in vitro* experiments the t-RNA and enzyme (180 μg) were incubated with other additions and the C^{14}-methyl donor at 37°C for one hour. The reactions were terminated by the addition of 20% TCA and the TCA-precipitated radioactivity was determined in a Tricarb Liquid Scintillation Spectrometer after thorough washing of the precipitate with cold TCA, ethanol and ethanol-ether mixture. In *in vivo* studies, animals were given a single dose of the labeled compound, intraperitoneally, and were sacrificed 6 hours later. The t-RNA fractions were isolated and counted as already described.

Results and discussion. Results of the *in vivo* experiments are presented in Table I. It can be seen that there is a greater incorporation of radioactivity from both $C^{14}H_3$-methionine and serine-3-C^{14} into t-RNA of vit. B_{12} deficient rats compared to the other two groups. This could be due to a greater dilution of the precursor (by a larger endogenous methyl pool in the latter two groups). While with $C^{14}H_3$-methionine there is no difference in t-RNA specific radioactivity, there is an increase with serine-3-C^{14} in the vit. B_{12} supplemented group compared to methionine-fed animals. This is evidently due to the established role of vit. B_{12} in methyl synthesis from one-carbon precursors. The results of the *in vivo* experiments do not, however, lead to any definite conclusions due to the fact that we are dealing with methylation of t-RNA which is already methylated to different degrees in the 3 groups, whereas the data on *in vitro* studies presented in Table II and III point to a possible vit. B_{12}-dependent pathway of methylation. From Table II it can be seen that there is a greater incorporation of radioactivity when the source of enzyme is from vit. B_{12}-fed animals with any of the 3 groups providing t-RNA substrate. Besides, the addition of either cyanocobalamin or DMBC-coenzyme enhances significantly the methylation of t-RNA by vit. B_{12}-deficient enzyme but not much stimulatory effect could be seen with the enzyme from vit. B_{12}-fed rat. Finally, it was found (Table III) that addition of cold S-adenosylmethionine does not dilute the radioactivity incorporated from serine-3-C^{14} into t-RNA of either rat liver or *Escherichia coli*. On the contrary, there is an enhancing effect observed. Vitamin B_{12} or B_{12} coen-

TABLE II. *In vitro* Methylation of Rat Liver t-RNA by $C^{14}H_3$-Methionine.

Enzyme from Group	Additions to complete system	Radioactivity t-RNA substrate (cpm) from Group I	II	III
I	———	48	136	76
	+ CN-B_{12} (200 γ)	124		
	+ DMBC-coenzyme (20 γ)	180		
II	———	126	220	200
	+ DMBC-coenzyme (20 γ)	166		
III	———	76	152	80

t-RNA was incubated with the enzyme at 37°C for 1 hr with the complete system consisting of: tris buffer pH 8.2, 100 μmoles; GSH (reduced), 100 μmoles; $MgCl_2$, 100 μmoles; PEP, 15 μmoles; PEP kinase, 3 μmoles; ATP, 2 μmoles and $C^{14}H_3$-methionine, 0.6 μc. After incubation the TCA-precipitated radioactivity was counted.

I—B_{12} deficient rats; II—B_{12} supplemented rats; III—B_{12} deficient rats + methionine.

TABLE III. Effect of Cold S-Adenosylmethionine on *in vitro* Incorporation of Serine-3-C^{14} into Rat Liver and *E. coli* t-RNA.

	Rat liver t-RNA	*E. coli* t-RNA
	Total counts (cpm)	
Complete system	86.7 ± 16.65	58.2 ± 11.59
Idem + S-adenosyl-methionine (100 μg)	126.53 ± 11.20	147.1 ± 16.98
	P = .01	P = .05

2 mg of t-RNA isolated from either rat liver or *E. coli* (Calbiochem) were incubated with methylating enzyme of rat liver and serine-3-C^{14} (0.1 μc) in the complete system. The TCA-precipitable total counts were determined at the end of 1 hr incubation.

Results are averages of 4 determinations ± S.D.

zyme stimulate t-RNA methylation probably as a result of conversion to methyl-B_{12} by the crude enzyme preparation used in the *in vitro* system. We have also carried out studies using chemically synthesised methyl-B_{12} as the methyl donor in an enzymatic reaction which forms methylated bases characterized by chromatography(16). Methyl-B_{12} could thus act as a methyl donor for t-RNA methylation as has been reported for homocysteine methylation by Guest *et al*(17,18) and also for the formation of methane and acetate in bacteria (19,20). Huennekens (21) has, however, recently postulated a mechanism of homocysteine methylation which does not involve the migration of the methyl group from methyl-B_{12}. Experimental data to support this mechanism have, however, not been published. The stimulatory effect of S-adenosylmethionine may then be similar to its yet unknown role in methyl transfer from methyl-THF A *via* methyl-B_{12} to homocysteine in methionine synthesis.

Summary. Methylation of t-RNA is studied both *in vivo* and *in vitro* in rat liver. Addition of either vitamin B_{12} or DMBC-coenzyme to the *in vitro* system improves significantly the rate of methyl incorporation from methionine-$C^{14}H_3$. Cold S-adenosylmethionine does not dilute the radioactivity incorporated from serine-3-C^{14}. An alternate pathway of t-RNA methylation involving methyl-B_{12} is suggested.

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