Natural Inhibitors of the Transfer Ribonucleic Acid Methylases*

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ABSTRACT: Extracts of adult organs contain an inhibitor of transfer ribonucleic acid (tRNA) methylases which is absent from extracts of foetal organs. An enzyme, tRNA N^2-guanine monomethylase, can be freed of the inhibitor by precipitation at pH 5. The inhibitor-free enzyme has an enhanced rate and capacity for methylation of a standard substrate. The inhibitor is active against the precipitated enzyme as well as against methylases from foetal and adult tissues. Inhibitors of lesser potency are present in extracts of a tumor, Novikoff hepatoma. The inhibitor from adult liver is nondialyzable, heat labile, and sensitive to digestion by trypsin. Ammonium ion has a slight stimulatory effect on crude enzyme extracts containing the inhibitor but has no effect on the activity of enzymes freed of the inhibitor.

The tRNA methylases (Fleissner and Borek, 1962), a group of enzymes which modify the structure of preformed tRNA by the addition of methyl groups at the macromolecular level, have unique characteristics. They are species and base specific (Srinivasan and Borek, 1963) and recognition between enzyme and substrate appears to involve not single nucleotides but long runs of them (Baguley and Staehelin, 1969). Because of the essential role that specific tRNA species and tRNA in general play in protein synthesis and its control, the study of the methylating enzymes, which confer structural and conformational individuality on tRNA molecules, is of interest. The modified bases in tRNA have been implicated in some functions. These involve the reaction with the aminoacyl-tRNA synthetase enzymes (Shugart *et al.*, 1968) and the ability to bind to ribosomes (Gefter and Russell, 1969). However, a complete understanding of all of the specific modifications of tRNA has not yet been reached.

The methylating enzymes have been shown to undergo alterations in a number of biological systems which are being subjected to shifts in regulatory mechanisms such as metamorphosing insects (Baliga *et al.*, 1965), colonizing slime mold (Pillinger and Borek, 1969), and differentiating bovine lens tissue (Kerr and Dische, 1968). Differences in the methylases in a number of malignant tumors as compared with normal tissue have also been shown (Borek, 1969). These differences include both an elevation in capacity to methylate and the specific bases methylated. Several investigators have reported differences in the level of methylating enzymes in organs of foetal, newborn, and adult animals (Hancock *et al.*, 1967; Kaye *et al.*, 1966; Simon *et al.*, 1967).

Whether the differences in these systems indicated by the *in vitro* assay of the methylases are an accurate reflection of the *in vivo* interactions has been questioned (Baguley and Staehelin, 1968a; Kaye and Leboy, 1968).

Kaye and Leboy (1968) have suggested that the *in vitro* differences between the methylating enzymes of tumor and normal tissues may merely be due to differences in the ionic conditions of the assays. Rodeh *et al.* (1967) and Zeleznick (1967) have reported a requirement for ammonium ions for optimal activity of the methylating enzymes in several systems though at vastly different concentrations.

McFarlane (1969) has reported that while ammonium ion stimulates the methylase activity in normal hamster liver, at the same concentration it does not affect the methylase activity from an adenovirus-12 induced tumor in hamsters. This finding implies a difference in the methylases of normal and tumor tissue. But analytical evidence of such differences has also been adduced. Viale *et al.* (1967) have found elevated levels of methylated bases in the tRNAs from six different human brain tumors and Bonney *et al.* (1969) have reported elevated levels of methylation in the tRNA extracted from tissues of Burkitt lymphoma.

Ribonuclease levels in tumor tissues compared with normal tissues are reported to be highly variable and they are the same in regenerating liver and normal liver (Reid, 1962), so it does not seem likely that nucleases are a contributing factor for the elevated levels of methylases found in tumor tissues.

The precise control mechanisms *in vivo* which regulate the activity of the tRNA methylases are obscure at present. An inhibitor of tRNA methylases in induced lysogenic *Escherichia coli* has been reported by Wainfan *et al.* (1965). We report here the existence of another such factor, a natural inhibitor which is present in adult organs of rabbit and rat and absent in their foetal counterparts.

Experimental Procedure

Materials. Pregnant New Zealand white rabbits were obtained from the Aldrich Rabbitry, Upton, Mass. Novikoff hepatoma grown on the omentum of rats from Holtzman Farms was supplied by Dr. Eric Hirschberg.

[^{14}C]Methyl-*S*-adenosylmethionine (specific activity 29.6 mCi/mmole; 15,000 cpm/mμmole) was purchased from Tracerlab, Inc., Waltham, Mass.

E. coli B and rabbit liver tRNAs were obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

Trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp., Freehold, N. J.

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TABLE I: Fractionation of tRNA Methylase Activity from Adult Rabbit Liver.[a]

Enzyme Fraction	Vol (ml)	Act. (U/ml)	Protein[b] (mg/ml)	Sp Act. (U/mg)	Recov (%)
100,000*g* supernatant	20	0.06	13.3	0.0045	100
pH 5 precipitate	20	0.44	2.1	0.21	730

[a] High-speed supernatant of adult rabbit liver homogenate was prepared as described in the text. A pH 5 precipitation was carried out on the high-speed supernatant and the precipitate was taken up in a volume of buffer equal to the original volume to avoid concentrating the enzyme. Assay conditions were those described in the text. Each assay contained 10 μg of *E. coli* B tRNA and 0.2 ml of enzyme solution. Incubation was for 1 hr at 37°. A unit of activity is defined as that amount of enzyme which incorporates 1 mμmole of [^{14}C]methyl into tRNA in 1 hr. [b] Protein was estimated by the method of Lowry *et al.* (1951).

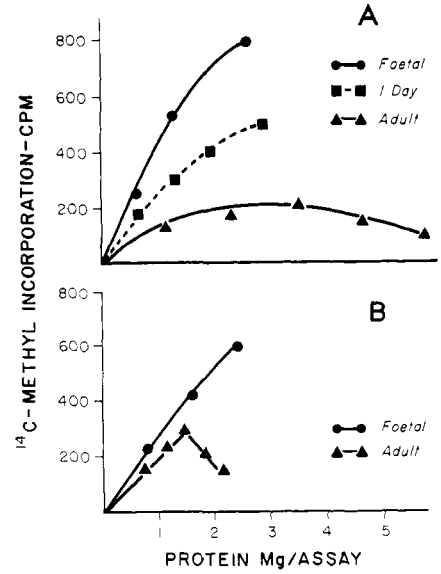

FIGURE 1: Methylation by extracts from foetal, newborn, and adult rabbit tissues. Assay tubes contained 10 μg of tRNA and were incubated for 30 min at 37°. (A) Methylation by high-speed supernatants from −10-day foetal, 1 day, and adult rabbit liver. (B) Methylation by high-speed supernatants from −10-day foetal and adult rabbit brain.

Assay of tRNA Methylase. The standard assay mixture contained: 50 μmoles of Tris-HCl buffer (pH 8.2), 5 μmoles of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 10 mμmoles of [^{14}C]methyl-*S*-adenosylmethionine, 1–30 μg of *E. coli* B tRNA, and varying amounts of enzyme in a total volume of 1 ml. The reaction was stopped by immersion in an ice bath and the tRNA was precipitated by the addition of 2 ml of 95% EtOH. The precipitate was washed thoroughly with 67% EtOH–0.5 M NaCl, was taken up in 0.2 M NH_4OH, and was transferred to a planchet for counting in a low-background gas-flow counter. Control reactions without tRNA were treated identically to determine the background level.

Preparation of Enzyme Solutions. Tissues were removed from the animals immediately after killing by decapitation and were either used fresh or stored at −20°. The tissue was homogenized in six volumes of 0.32 M sucrose–0.002 M $MgCl_2$–0.005 M 2-mercaptoethanol. The homogenate was centrifuged successively at 30,000*g* for 10 min and 105,000*g* for 60 min. The high-speed supernatant was used for the crude enzyme assay. In the case of organs of adult animals, the high-speed supernatant also contains a natural inhibitor of the tRNA methylases. An enzyme preparation relatively free from inhibitor was obtained by the following procedure. The high-speed supernatant was brought to pH 5.0 by the addition of 1 N acetic acid and centrifuged immediately at 10,000*g* for 10 min. The supernatant was removed and neutralized and was used as a source of inhibitor. The precipitate was washed once with 0.32 M sucrose–0.002 M EDTA (pH 5.0) and was then taken up in 0.01 M Tris-HCl buffer (pH 8.2) containing 0.002 M EDTA and 0.005 M 2-mercaptoethanol. This solution was used as a source of inhibitor-free enzyme.

Results

Comparison of Methylase Activity in Foetal, Newborn, and Adult Rabbit Organs. In Figure 1 comparisons of enzyme activities of high-speed supernatants prepared from extracts of the liver of −10-day foetal rabbit, newborn rabbit, and adult rabbit and from extracts of the brain of −10-day foetal and adult rabbit are presented. Assay of tRNA methylase activities in the crude supernatants revealed that the extract from foetal liver has a fourfold greater capacity to methylate than the extract from adult liver. This was also true in the case of brain tissue where the foetal extract had a methylating capacity more than twice as great as the extract from adult brain. In the case of the liver from newborn animals, the activity in extracts was intermediate between foetal and adult extracts. These results confirm the findings of Hancock *et al.* (1967) and Simon *et al.* (1967). Another characteristic which can be seen in the case of extracts from adult liver and brain is a decrease of enzyme activity beyond a certain concentration of protein. This could imply the presence of an inhibitor whose action depends upon its absolute concentration.

Separation of a Natural Inhibitor from tRNA Methylase Activity. The admixture of foetal and adult liver extracts revealed that the adult extract could lower the methylase activity of foetal extracts, which implied the presence of an inhibitor.

In order to separate the inhibitor from the methylase activity, the pH 5 precipitation of the enzyme described above was used. A methylase activity of increased capacity was recovered from the precipitate, while the reneutralized supernatant contained an inhibitor of this activity. The data in Table I revealed that the pH 5 precipitation removes an inhibitor since greater than 100% of the original enzyme activity is recovered. The enzyme redissolved from the pH 5 precipitate was unstable, losing 10–20% activity overnight whether stored at 4 or −20°.

Identification of the Enzyme in the pH 5 Precipitate. [^{14}C]-Methyl-tRNA was isolated from a large-scale methylation

TABLE II: Effect of Increased Substrates on Inhibition.[a]

Enzyme (mg/ml)	Inhibitor (mg/ml)	tRNA (μg)	*S*-Adenosyl-methionine (mμmoles)	$\mu\mu$moles of [^{14}C]-Methyl Incorpd	Inhibn (%)
0.3		10	5	35.5	
0.3	3.1	10	5	22.7	36.1
0.3		100	5	72.3	
0.3	3.1	100	5	45.4	37.2
0.3		10	25	38.5	
0.3	3.1	10	25	24.7	35.9
0.6		10	5	43.0	
0.6	3.1	10	5	43.1	None

[a] Reaction conditions were standard but substrate concentrations were varied. Total volume was 1 ml.

using the enzyme activity found in the pH 5 precipitate. The tRNA was subjected to enzyme digestion and column chromatography according to the method of Hall (1965). The only methylated derivative found was N^2-methylguanosine. The radioactive product cochromatographed with an authentic sample of N^2-methylguanosine (Cyclo Chemical Co.) in three solvent systems (1-butanol–water–concentrated ammonium hydroxide (86:14:5), 1-butanol–98% formic acid–water (77:10:13), and isopropyl alcohol–concentrated hydrochloric acid–water (680:170:144)).[1]

Activity Spectrum of Inhibitor from Adult Rabbit Liver. The reneutralized pH 5 supernatant was used as a source of inhibitor in all assays and it was assayed against the adult liver enzyme recovered from the pH 5 precipitate, the N^2-guanine monomethylase, as well as against mixtures of enzymes.

As seen from the data in Figure 2 the inhibitor from adult tissue was active against enzyme from foetal rabbit liver as well as the enzyme of adult liver.

The inhibitor from adult rabbit liver was also tested against methylases from adult and foetal rabbit brain, rat liver, and Novikoff hepatoma and was found to be effective in all cases. No attempt was made to quantitate its relative effectiveness on the different systems, but 90% inhibition could be reached in all cases.

Nature of the Inhibitor. In the methylation reaction involving the N^2-guanine monomethylase the inhibitor appears to act on the enzyme itself rather than on either of the substrates. The data in Table II show that increasing the concentration of substrates, tenfold for tRNA or fivefold for *S*-adenosylmethionine, does not overcome the inhibition, while increasing the level of enzyme can relieve the inhibition completely.

The possibility that the inhibitor is a nuclease or acts on the tRNA in some other manner was ruled out by preincubation of tRNA with the inhibitor after which the inhibitor was heat inactivated and enzyme and *S*-adenosylmethionine were added. No difference in the degree of methylation of untreated tRNA and tRNA preincubated with inhibitor was observed (Table III).

The inhibitor was also incubated with *S*-adenosylmethionine and cleavage was tested for by the method of Gefter *et al.* (1966). No difference was observed between controls and inhibitor-treated preparations.

Analysis of kinetic studies of free enzyme and inhibited enzyme (Figure 3) revealed a pattern indicating the presence of a noncompetitive inhibitor. The inhibitor also affects another parameter of the methylating enzymes, the extent of methylation. Methylating enzymes from a given source will methylate heterologous tRNA substrates only to a characteristic level for each tRNA. The N^2-guanine monomethylase free from inhibitor can methylate *E. coli* B tRNA to an extent of 10.7 mμmoles/mg of tRNA. The inhibitor not only decreases the rate of methylation by this enzyme but also lowers the level to which it can methylate in proportion to the amount of inhibitor added.

The inhibitor was nondialyzable and could be inactivated by heating at 100° for 1 min or by incubation with trypsin (50 μg/ml) for 1 hr at 37°. These observations imply that the inhibitor is a protein.

Methylation of Homologous tRNA. Under normal circumstances, tRNA from a given source is fully methylated with respect to the methylating enzymes from the same source and will not serve as a methyl acceptor in an *in vitro* assay. tRNA from a heterologous source must be used as a substrate. Rabbit liver tRNA was tested as a substrate with the inhibitor-free N^2-guanine monomethylase and with extracts from foetal rabbit liver. The results given in Table IV demonstrate that the inhibitor-free extracts from adult liver and extracts of foetal liver can hypermethylate the homologous adult rabbit liver tRNA to a measurable extent.

[1] The finding of only a single methylating activity in the pH 5 precipitate raises a question as to the whereabouts of the other base-specific methylases known to exist in mammalian tissues. While the method used here for separation of inhibitor and enzyme was chosen for its simplicity, the inhibitor can also be separated by other methods including ammonium sulfate fractionation or chromatography on DEAE-cellulose. If the reneutralized pH 5 supernatant, from which the N^2-guanine monomethylase has been removed, is subjected to DEAE-cellulose chromatography, at least four distinct methylating activities can be detected. These methylases then are present in the high-speed supernatant but their activity cannot be demonstrated unless they are separated from the inhibitor by some method other than a pH 5 precipitation.

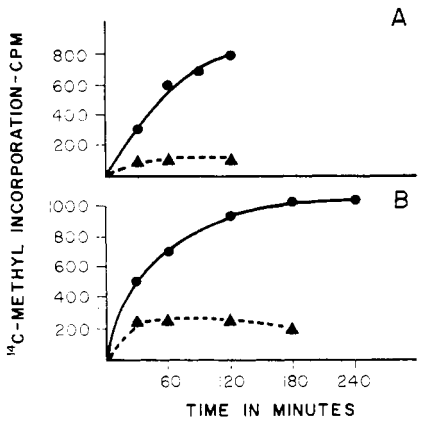

FIGURE 2: Effect of inhibitor on foetal and adult methylase. Assay tubes contained 10 μg of tRNA/ml. (A) (●—●) 100,000g supernatant of −10-day foetal rabbit liver, protein concentration of 2.1 mg/ml. (▲—▲) 100,000g supernatant of −10-day foetal rabbit liver (protein 2.1 mg/ml) with inhibitor from adult rabbit liver (protein 4.5 mg/ml). (B) (●—●) Inhibitor-free enzyme from adult rabbit liver, protein concentration 0.75 mg/ml. (▲—▲) Inhibitor-free enzyme (protein 0.75 mg/ml) with inhibitor from adult rabbit liver (protein 4.5 mg/ml).

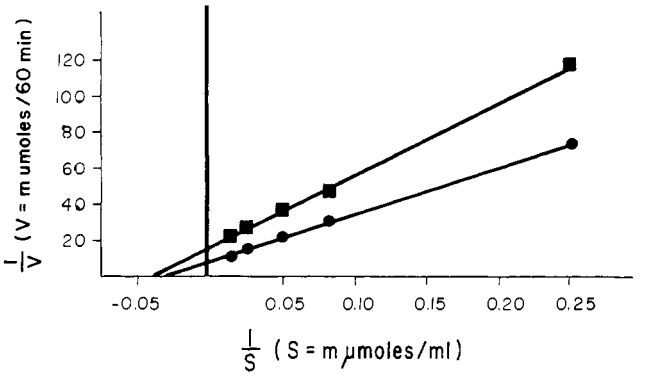

FIGURE 3: Lineweaver–Burk plot of methylation kinetics for free enzyme and enzyme with inhibitor. S refers to tRNA concentration; V refers to [^{14}C]methyl incorporation. (●—●) Inhibitor-free enzyme (1.1 mg/ml). (■—■) Inhibitor-free enzyme (1.1 mg/ml) with inhibitor from adult rabbit liver (1.8 mg/ml).

Presence of an Inhibitor in Tumor Tissue. The findings of elevated levels of methylation in a variety of tumor tissues (Borek, 1969) raised the question whether this might be due merely to an absence of a natural inhibitor. Extracts of Novikoff hepatoma were therefore examined.

When a 100,000g supernatant from Novikoff hepatoma was fractionated by pH 5 precipitation, the methylating activity of the precipitate had the same initial rate of methylation as the crude supernatant, but its capacity to methylate was twice as great. The reneutralized pH 5 supernatant itself also had a measurable methylase activity. However, the two activities were not additive. When the extracts from the pH 5 precipitate and the reneutralized pH 5 supernatant were reconstituted in their original proportions the methylase activity dropped to its original value (Table V).

As a control the same experiment was carried out using normal rat liver. The results are included in Table V and can be seen in Figure 4 where the time course of methylation by extracts of Novikoff hepatoma and normal rat liver is shown. Extracts of the rat liver behaved as did extracts of rabbit liver with an increased activity in the precipitated enzyme and no detectable activity in the reneutralized supernatant. The hepatoma extracts had a greater capacity to methylate than any of the extracts from normal tissue.

The identities of the specific bases methylated by the different fractions from Novikoff hepatoma and rat liver have not been determined.

Effect of Ammonium Ion. The effect of ammonium ion on the N^2-guanine monomethylase from adult rabbit liver in the presence and absence of inhibitor was also examined. Ammonium acetate was used at a final concentration of 0.36

TABLE III: Effect of Inhibitor on tRNA.[a]

Substrate	$\mu\mu$moles of [^{14}C]-Methyl Incorpd I	II
tRNA, no inhibitor	44.2	32.5
tRNA, preincubated with inhibitor and then heat inactivated	45.9	31.5
tRNA, preincubated with inhibitor and not heat inactivated	20.5	15.0

[a] *E. coli* B tRNA (20 μg) was preincubated for 1 hr at 37° with inhibitor at a concentration (3.3 mg of protein/ml) which was known to give 50% enzyme inhibition. The inhibitor was then inactivated by heating the incubation mixture at 100° for 5 min. As a control tRNA without inhibitor was treated in the same way. Enzyme (0.25 mg of protein/ml) and [^{14}C]methyl-*S*-adenosylmethionine were then added and incubation was carried out for 60 min at 37°. Data from two separate experiments are shown.

TABLE IV: Methylation of Rabbit Liver tRNA by Homologous Enzymes.[a]

Enzyme Source	$\mu\mu$moles of [^{14}C]Methyl Incorpd
Adult rabbit liver, 100,000g supernatant	1.5
Adult rabbit liver, pH 5 precipitate (N^2-guanine monomethylase)	16.6
−10-day foetal rabbit liver, 100,000g supernatant	18.3

[a] Standard assay conditions were used with 400 μg of adult rabbit liver tRNA as substrate. Protein concentrations (mg) in the assays were: adult rabbit liver 100,000g supernatant, 2.3; adult rabbit liver N^2-guanine monomethylase, 0.88; −10-day foetal rabbit liver 100,000g supernatant, 2.2. Incubation was for 120 min at 37°.

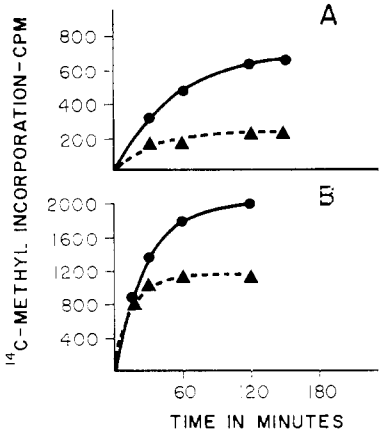

FIGURE 4: Methylation by extracts from Novikoff hepatoma and by extracts from normal rat liver. Assays contained 10 μg of tRNA and were incubated at 37°. (A) (▲--▲) 100,000g supernatant of normal rat liver, protein concentration 2.5 mg/ml. (●—●) Inhibitor-free enzyme from pH 5 precipitate, protein concentration 0.42 mg/ml. (B) (▲--▲) 100,000g supernatant from Novikoff hepatoma, protein concentration 1.3 mg/ml. (●—●) Inhibitor-free enzyme from pH 5 precipitate, protein concentration 0.56 mg/ml. Note the difference in scale of [^{14}C]methyl incorporation between A and B.

TABLE V: tRNA Methylase Activity in Novikoff Hepatoma.[a]

Enzyme Fraction	Vol Assayed (ml)		$\mu\mu$moles of [^{14}C]Methyl Incorpd	
	Hepatoma	Rat Liver	Hepatoma	Rat Liver
100,000g supernatant	0.2	0.3	53	11
pH 5 precipitate	0.2	0.3	93	30
Reneutralized pH 5 supernatant	0.2	0.3	18	0
pH 5 precipitate + pH 5 supernatant	0.2 + 0.2	0.3 + 0.3	57	10

[a] The enzyme fractions were prepared as described in the text with the pH 5 precipitate taken up in a volume of buffer equal to the original volume. Standard assay conditions were used with 10 μg of *E. coli* B tRNA as substrate. Incubations were for 30 min at 37°. Protein concentration in the hepatoma 100,000g supernatant was 6.6 mg/ml; in the rat liver 100,000g supernatant it was 8.3 mg/ml.

M as this is the concentration reported by Rodeh *et al.* (1967) and by Kaye and Leboy (1968) which yields maximal stimulation with methylases from mammalian sources. The results can be seen in Figure 5 which shows the time course of methylation by free enzyme, with and without ammonium ion, and enzyme plus inhibitor, with and without ammonium ion. The presence of ammonium ion proved to be slightly inhibitory to the separated N^2-guanine monomethylase but it did have a stimulatory effect in the presence of inhibitor.

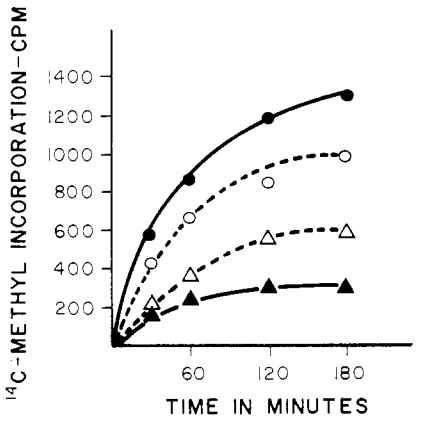

FIGURE 5: Effect of ammonium ion on inhibitor-free enzyme and enzyme with inhibitor. Assays contained 10 μg of tRNA. Inhibitor-free enzyme from adult rabbit liver, protein concentration 0.9 mg/ml: (●—●) standard assay conditions; (○--○) standard assay conditions plus 0.36 M ammonium acetate. Inhibitor-free enzyme from adult rabbit liver, protein concentration 0.9 mg/ml with adult liver inhibitor, protein concentration 3.8 mg/ml: (▲—▲) standard assay conditions; (△--△) standard assay conditions plus 0.36 M ammonium acetate.

Thus the mode of action of NH_4^+ could be a partial reversal of inhibition.

The effect of 0.36 M ammonium acetate was also tested with the enzyme fractions from Novikoff hepatoma and no stimulation in any of the fractions was found. This finding parallels the observation of McFarlane (1969) who, as stated earlier, found no stimulatory effect of NH_4^+ in extracts of adenovirus-12 tumors.

Discussion

The results presented here confirm reports of elevated tRNA methylase levels (Kaye *et al.*, 1966; Hancock *et al.*, 1967; Simon *et al.*, 1967) in foetal tissues as compared with their adult counterparts. The presence of a natural inhibitor in adult organ extracts has been demonstrated and the removal of this inhibitor leads to the recovery of an enzyme activity approaching that of foetal tissue. The procedure used here, pH 5 precipitation, separates an N^2-guanine monomethylase from the inhibitor. The inhibitor is active against this enzyme and the other base-specific enzymes in adult rabbit liver. It is also active against foetal rabbit liver extracts as well as foetal and adult rabbit brain extracts and extracts from rat liver and Novikoff hepatoma. The inhibitor appears to be a nondialysable protein which acts on the enzyme rather than the reaction substrates. The presence of natural inhibitors in adult rat liver and in Novikoff hepatoma has also been demonstrated.

The effect of ammonium ion on the inhibition of adult rabbit liver enzyme was investigated and it was found to partially reverse the inhibition while having no stimulatory effect on the free N^2-guanine monomethylase. In the case of methylase activity from Novikoff hepatoma no effect of ammonium ion was detected.

Our studies do not exclude the possibility of the presence of multiple inhibitors. It is possible that base-specific inhibitors

exist which could be separated by more refined fractionations. Neither can the existence of an inhibitor with varied potency toward the base specific enzymes be ruled out at present. The inhibitor found in Novikoff hepatoma would appear to be somewhat different from that of normal tissue as it does not exhibit the same response to ammonium ion.

The question of ion effects is a complicated one, made more so by the fact that the experiments reported in the literature were performed on a mixture of methylating enzymes. The exact ionic and pH requirements for each of the base-specific enzymes may well be different. For instance, Baguley and Staehelin (1968a) report a requirement of ammonium ion by purified 1-adenine methylase while the N^2-guanine monomethylase separated here does not show such a requirement. The existence of a natural inhibitor shown here must also be taken into account when interpreting data obtained from crude multienzyme systems. Separation of an inhibitor probably accounts for the observation by Rodeh *et al.* (1967) that the pattern of methylation from rat liver extracts changed as the enzymes were purified. Baguley and Staehelin's (1968b) finding that purified 1-adenine methylase from rat liver could methylate to a greater extent than a crude extract also was probably due to the elimination of an inhibitor.

However, it is likely that the enzyme and inhibitor present in extracts function in concert *in vivo* as well, because there is good concordance between enzyme activity measured *in vitro* and analytical data on isolated tRNAs. Thus numerous investigators have reported elevated enzyme capacity in crude tumor extracts (Borek, 1969) and the analysis of tRNAs from nine different tumors has revealed higher than normal frequency of methylated bases (Bergquist and Matthews, 1962; Viale *et al.*, 1967; Bonney *et al.*, 1969). These findings support the validity of the *in vitro* enzyme studies.

Whether the high methylase level found in foetal extracts also reflects a higher degree of methylation in tRNA of foetal tissues will also have to be confirmed by actual analysis of the tRNA. However, the finding that foetal extracts can methylate tRNA from adult rabbit suggests that adult tRNA may be hypomethylated with respect to foetal tRNA. Differences in the actual tRNA species in adult and foetal tissues have been observed. Lee and Ingram (1967) have found both qualitative and quantitative differences in the methionyl-tRNAs of immature red blood cells from chick embryo and of reticulocytes from adult chickens. It has not been determined what the actual difference is between foetal tRNA species and adult tRNAs, but it is possible that methylation could play a role in these alterations.

The biological significance of these differences between foetal and adult tissues is not clear. However, there is increasing evidence that tRNA may be involved in regulation of protein synthesis at the translational level, both in bacteria (Smith *et al.*, 1966; Carbon *et al.*, 1966; Silbert *et al.*, 1966) and in higher organisms (Wainwright and Wainwright, 1967), and that changes in tRNA molecules can lead to alterations in their function in translation (Fitler and Hall, 1966; Capra and Peterkofsky, 1968; Gefter and Russell, 1969).

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