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Absence of a Natural Inhibitor of the tRNA Methylases from Fetal and Tumor Tissues

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ABSTRACT **The inhibitor of tRNA methylases of normal adult tissues has been resolved into two fractions, a high molecular weight protein and a low molecular weight (<700) component. The high molecular weight component is absent from embryonic and tumor tissues, both of which are known to possess high tRNA methylase capacity.**

Normal adult tissues contain inhibitors of the enzymes that methylate tRNA (1). I report here on the purification and separation of the inhibitor from normal tissue into two fractions, a high molecular weight protein and a low molecular weight component. Neither of the components alone inhibits the tRNA methylases, but in concert they restore the full inhibitory power of the original extracts of adult tissue. Extracts of all tumors examined exhibit aberrantly high tRNA methylase capacity (2), as do extracts of fetal tissues (3, 4), so that it was of interest to determine whether the inhibitor complex functions in these tissues. Extracts of three different tumors, Novikoff hepatoma, a Morris hepatoma, and Ehrlich ascites cells, as well as an extract of fetal rabbit liver, were examined. All the tissues, tumor and fetal, were found to lack the high molecular weight component of the tRNA methylase inhibitor complex found in adult tissues.

MATERIALS AND METHODS

Adult New Zealand white rabbits were obtained from Creagar's Rabbit Ranch, Colo. Fetal rabbit liver from the third week of development was supplied by Pel-Freez Biologicals, Inc. Novikoff hepatoma grown on the omentum of rats was kindly supplied by Dr. Beard of Albert Einstein Medical School, N.Y. Morris hepatoma, 5123 c, gen. 79, grown on the hind legs of rats, was provided by Dr. Jean-Pierre Jost of the National Jewish Hospital, Denver, Colo. Ehrlich ascites tumor cells, collected from the peritoneal cavity of mice 6–8 days after inoculation, were the gift of Dr. Walden Roberts of this department.

Assay for tRNA methylase

The standard assay conditions have been described (1). *S*-adenosyl-[methyl-^{14}C]-L-methionine (5–6 $\times$ 10^7 cpm/μmol) was purchased from International Chemical and Nuclear Corp. and *Escherichia coli* B tRNA was obtained from General Biochemicals, Inc. Each assay contained 50 mM Tris·HCl (pH 8.2)–5 mM $MgCl_2$–5 mM β-mercaptoethanol. The reaction was stopped by the addition of an equal volume of cold 10% trichloroacetic acid (TCA). After 10 min at 4°C, the insoluble material was collected by filtration through glass fiber filters (Whatman GF/C, 2.4-cm diameter), washed with six 4-ml aliquots of 5% TCA, and was counted in a liquid scintillation counter (Nuclear-Chicago). A unit of enzyme activity is that amount of enzyme that incorporates 1 nmol of [^{14}C]methyl into tRNA in 1 hr.

Separation of the inhibitor

Homogenization and High-speed Centrifugation. The tissues to be investigated were homogenized in 6 vol of 10 mM Tris·HCl (pH 7.4)–10 mM NaCl–1.5 mM $MgCl_2$. The homogenate was centrifuged successively for 10 min at 30,000 $\times$ *g* and 60 min at 105,000 $\times$ *g*.

pH 5 Precipitation. The high-speed supernatant fluid was brought to pH 5.0 by the careful addition of 1 N acetic acid and was centrifuged immediately for 10 min at 10,000 $\times$ *g*. The pH 5 supernatant was neutralized with 1 M Na_2CO_3. The precipitate was extracted with 0.05 M potassium phosphate, pH 6.0, and was centrifuged for 10 min at 30,000 $\times$ *g*. The extract contained the pH 5-insoluble enzyme, which was then precipitated by adding 3 g of $(NH_4)_2SO_4$ for each 10 ml of solution. The enzyme was stable in this form when stored at 4°C.

DEAE-Cellulose Fractionation. The reneutralized pH 5 supernatant was applied to a DEAE-cellulose column (1 ml of column volume for each ml of solution) equilibrated with 50 mM Tris·HCl, pH 8.0–5 mM β-mercaptoethanol. The fraction containing the activity inhibitory toward the tRNA methylases was not absorbed by the column and could be eluted with the starting buffer. Any tRNA methylases remaining in the original pH 5 supernatant (1) were absorbed to the DEAE-cellulose.

Gel Filtration on Sephadex G-25. The inhibitor fraction washed off the DEAE-cellulose column was put through a Sephadex G-25 (coarse) column equilibrated with 50 mM Tris·HCl, pH 8.0–5 mM β-mercaptoethanol. The elution pattern, measured by absorbance at 280 nm, for different tissues showed a variable number of peaks, but never less than two (see Fig. 1).

RESULTS

Separation of the natural inhibitor of the tRNA methylases into two components

As reported (1), when a high-speed supernatant extract from normal adult rabbit tissues is precipitated at pH 5, a tRNA methylase activity—essentially N^2-guanine monomethylase—is found in the fraction precipitated at pH 5, while an inhibitor of this activity remains in the pH 5 supernatant. The

inhibitor can be freed from the other methylase enzymes by passing it through DEAE-cellulose.

If the solution containing the methylase-free inhibitor is next fractionated by column chromatography on Sephadex G-25, an elution pattern is obtained such as the one in Fig. 1. When the peak fractions obtained by this method were tested separately for inhibitory activity toward the pH 5 enzyme fraction, no inhibition could be detected. However, if peak I and peak II were combined, the usual inhibition of the pH 5 enzyme resulted. Such an experiment is shown in Table 1 where it can be seen that addition of either the high molecular weight protein or the low molecular weight fraction alone does not inhibit the pH 5 enzyme; however, when both are added the enzyme is inhibited. These results indicate that the inhibitor consists of at least two components: a high molecular weight and a low molecular weight component both of which are required for inhibition;

There was a proportionality between the amount of protein in peak I and the protein concentration of the original 100,000 × *g* supernatant. When the optimal enzyme activity of the 100,000 × *g* supernatant is compared to the activity of the pH 5 enzyme derived from it, the increase in enzyme activity is from 2- to 2.5-fold, indicating that, at a protein concentration of 0.5–0.7 mg per 0.2 ml assay, the inhibition of the enzyme in the 100,000 × *g* supernatant extract is 50–60%. In the experiment shown in Table 1, addition of an amount of peak I that contained 0.35 mg of protein, which represented 65% of the protein content of the 100,000 × *g* supernatant, resulted in 33% inhibition of the enzyme. This represents about 60% of the calculated inhibition of the enzyme in the original 100,000 × *g* supernatant. The low molecular weight fraction cannot as yet be quantitated, so in these experiments an aliquot equal to the volume of the source of the high molecular weight fraction was added.

Properties of the two inhibitor components

The high molecular weight component was sensitive to trypsin and to heating at 100°C for 1 min. It could be precipitated with $(NH_4)_2SO_4$ at a concentration between 30 and 50% saturation. Its elution pattern on Sephadex G-100 and Sephadex G-75 indicated that its molecular weight lies between 75,000 and 100,000.

The low molecular weight component was not inactivated by heating at 100°C for 10 min and its elution pattern on Sephadex G-10 indicated that it is below 700 in molecular weight.

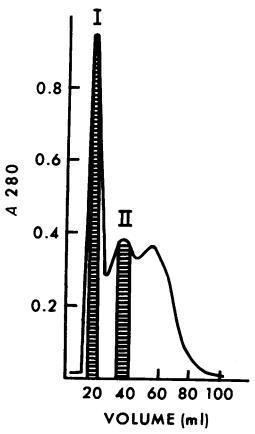

FIG. 1. Fractionation of the natural inhibitor of the tRNA methylases on Sephadex G-25. 3 ml of the methylase-free inhibitor of adult rabbit liver, prepared as described in the text, was passed through a Sephadex G-25 (coarse) column (1.5 × 30 cm) equilibrated with 50 mM Tris·HCl (pH 8.0)–5 mM β-mercaptoethanol. The solid line shows absorbance at 280 nm, while the hatched areas mark the high molecular weight (I) and low molecular weight (II) fractions tested for inhibitory activity (see Table 1).

TABLE 1. *Requirement for two factors for the inhibition of N^2-guanine monomethylase of adult rabbit liver*

Additions	[^{14}C]methyl incorporation (pmol)	% inhibition
None	39.0	...
High molecular weight fraction	38.4	0
Low molecular weight fraction	39.1	0
Both fractions	26.2	33

The incubation was performed under standard conditions in a volume of 0.2 ml with 100 μg of tRNA for 60 min. Enzyme protein concentration was 75 μg. Protein concentration in the assays containing the high molecular weight fraction was 0.35 mg. A volume (0.06 ml) of the low molecular weight fraction equal to the volume of the high molecular weight fraction was added where appropriate.

Interaction of a tRNA methylase with the high molecular weight inhibitory component

If the enzyme precipitated at pH 5—N^2-guanine monomethylase—and the high molecular weight inhibitor component are chromatographed individually on Sephadex G-200, the high molecular weight inhibitor component elutes later than the pH 5 enzyme (Fig. 2*A*, *B*). If the pH 5 enzyme and the high molecular weight component are mixed together, then cochromatographed on Sephadex G-200, enzyme activity is detected at an earlier elution volume than that of the pH 5 enzyme alone, and inhibitory activity cannot be detected at all (Fig. 2*C*). The enzyme activity at the new elution position can be inhibited by the low molecular weight inhibitor alone. These results indicate that the high molecular weight component that can be separated from the enzyme at pH 5 reacts with or binds to the enzyme at neutral or slightly alkaline pH so strongly that the two cannot be separated by gel filtration, these findings also confirm the requirement for two components for inhibition.

Absence of the high molecular weight component from fetal tissue

As reported (3, 4), the tRNA methylase capacity in fetal tissues is greater than that in their adult counterparts. I now report that this is due, in part, to the absence of the inhibitor found in normal adult tissues. Fetal rabbit liver was examined to determine whether the lack of inhibitory activity is due to the absence of one or both of the inhibitor components found in adult tissues. The results summarized in Table 2 indicate that the high molecular weight inhibitor from adult extracts can cause inhibition by itself of tRNA methylase activity in a high-speed supernatant extract of fetal rabbit liver. Addition of the low molecular weight component to the same extract

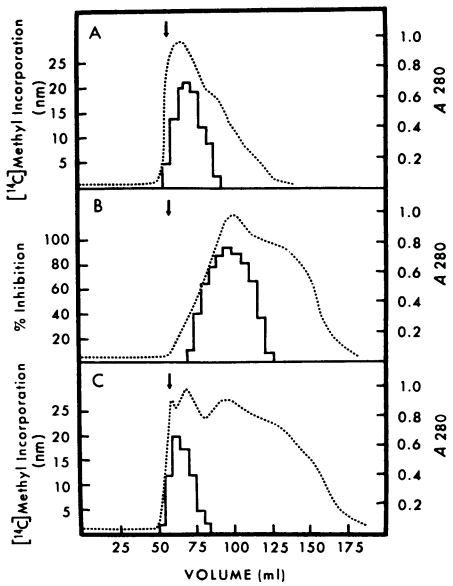

FIG. 2. Gel filtration on Sephadex G-200 of N^2-guanine monomethylase and the high molecular weight inhibitor component of adult rabbit liver. (*A*) 5 ml of the solution of N^2-guanine monomethylase precipitated at pH 5 from adult rabbit liver extract was passed through a Sephadex G-200 column (2.5 × 45 cm) equilibrated with the buffer of Fig. 1. The dotted line is A_{280}, while the bar graph shows enzyme activity, expressed as pmol [^{14}C]methyl incorporated into tRNA in 1 hr. Enzyme assays were performed on 0.1-ml aliquots in a total reaction volume of 0.3 ml. (*B*) 5 ml of a solution of the high molecular weight inhibitor component of adult rabbit liver was passed through the same Sephadex G-200 column as in (*A*). In this case the bar graph shows % inhibition of the N^2-guanine monomethylase as assayed by incorporating 0.2-ml aliquots into reaction mixtures containing 0.05 unit of enzyme activity and the low molecular weight component in a total volume of 0.3 ml. Incubation was for 60 min. (*C*) N^2-guanine monomethylase and the high molecular weight component were mixed, and 10 ml of the solution was passed through the Sephadex G-200 column. Enzyme activity was assayed as in (*A*). The high molecular weight component was assayed as in (*B*) but it could not be detected separately. However, the enzyme peak could now be inhibited by the low molecular weight component alone, which was not the case in (*A*), which indicated that the high molecular weight inhibitor component was now being eluted with the N^2-guanine monomethylase. The small arrow in the three panels indicates the void volume of the Sephadex G-200 column.

does not cause any inhibition. This finding implies the absence of the high molecular weight component and the presence of the smaller one at that stage in the development of the fetus.

Nature of the inhibitor in some tumor tissues

The tRNA methylase activity in a number of tumor tissues is known to be elevated compared to their normal counterparts (2). I have examined three different tumors to determine whether the elevated methylase capacity might be due to the absence of the normal adult inhibitors. The tumors were Novikoff hepatoma, a minimal deviation Morris hepatoma, and Ehrlich ascites cells. The elevated methylase capacity of the Novikoff hepatoma has been reported (5). The methylase activities of the other two tumor tissues, which had not previously been reported, were found to be elevated (unpublished observations).

TABLE 2. *Effect of the inhibitor components of adult rabbit liver on the tRNA methylases of fetal rabbit liver*

	[^{14}C]methyl incorporation (pmol)	% inhibition
Fetal enzyme	36.8	...
+ Adult high molecular weight inhibitor component	20.1	46
+ Adult low molecular weight inhibitor component	42.2	0
+ Both inhibitor components	11.2	70

Incubation was in a volume of 0.3 ml with 200 μg of tRNA for 60 min. Each assay contained 1 mg of fetal protein from a high-speed supernatant extract of three-week fetal rabbit liver. 0.7 mg of high molecular weight inhibitor protein was used in the assays. An amount of low molecular weight component was added sufficient to cause 50% inhibition in combination with the high molecular weight component of an adult enzyme system. To avoid the possibility of operating at marginal activities of enzymes, the total activity of the adult system was twice that of the fetal.

When the three tumor tissues were subjected to the procedure used to separate the normal adult inhibitor into its two components, a pattern different from that of the extracts of normal adult tissue was found. After the extracts had been carried through the G-25 gel filtration step, the enzymes precipitated at pH 5 from the tumor tissues were not inhibited by the combined fractions. Occasionally, inhibition by the high molecular weight fraction by itself was observed, but this has been traced to nuclease activity sometimes associated with that protein fraction from tumor tissue. The pH 5 enzymes from normal rat and mouse liver could not be inhibited by either Fraction I or II alone (from the tumor tissues) or by the combined fractions. However, if the pH 5 enzyme from normal rat liver or mouse liver was combined with their homologous high molecular weight inhibitor components, they were then susceptible to inhibition by the low molecular weight fractions from the tumor tissues (Tables 3 and 4).

It appears, then, that the tumor tissues contain the low molecular weight inhibitor but lack the high molecular weight component. This was further tested by incubating high-speed supernatant extracts from tumor tissues which had not been subjected to any fractionation with the high molecular weight and low molecular weight inhibitors from normal tissue. The results, shown in Table 5, indicate that addition of the high molecular weight component from normal adult rabbit liver caused inhibition of the methylases in all three tumor extracts. This confirms that the high molecular weight inhibitor is either absent, or present only in undetectable amounts, in tumor tissues and was not merely lost in the fractionation procedures.

DISCUSSION

Extracts of tumor and fetal tissues are known to contain very high tRNA methylase capacity compared to their normal adult counterparts (2–5). The discovery of natural inhibitors of the tRNA methylases, which control the enzyme activity in normal tissue, suggested a possible source of the high

TABLE 3. *Assay* for the high and low molecular weight inhibitor components in normal rat liver, Novikoff hepatoma, and Morris hepatoma*

Addition	Rat liver pH 5 enzyme			Novikoff hepatoma pH 5 enzyme			Morris hepatoma pH 5 enzyme		
	Alone	Plus high MW fraction	% inhibition	Alone	Plus high MW fraction	% inhibition	Alone	Plus high MW fraction	% inhibition
None	89.2	91.2	...	42.3	45.5	...	52.8	59.1	...
Rat liver low MW fraction	87.5	45.5	50	47.8	43.3	5	54.3	55.7	6
Novikoff hepatoma low MW fraction	88.9	50.2	45	49.1	45.9	0	56.2	56.9	4
Morris hepatoma low MW fraction	89.7	67.5	26	48.2	46.2	0	54.7	58.8	0

Incubations were with pH 5 tRNA methylase in a volume of 0.3 ml with 200 μg of tRNA for 60 min. The pH 5 enzymes were recombined with their homologous high and various low molecular weight fractions in the original proportions, i.e., dilutions of each solution were the same. Protein concentrations in the assays were: rat liver pH 5 enzyme, 200 μg; rat liver high molecular weight fraction, 250 μg; Novikoff hepatoma pH 5 enzyme, 15 μg; Novikoff hepatoma high molecular weight fraction, 30 μg; Morris hepatoma pH 5 enzyme, 100 μg; and Morris hepatoma high molecular weight fraction, 100 μg.

* Values are in picomoles [^{14}C]methyl incorporated.

TABLE 4. *Assay* for the high and low molecular weight inhibitor components in normal mouse liver and Ehrlich ascites cells*

Addition	Mouse liver pH 5 enzyme			Ehrlich ascites cells pH 5 enzyme		
	Alone	Plus high MW fraction	% inhibition	Alone	Plus high MW fraction	% inhibition
None	73.7	75.7	...	42.4	45.6	...
Mouse liver low MW fraction	74.9	52.2	31	43.1	44.9	0
Ascites cells low MW fraction	73.2	49.2	35	42.7	45.3	0

Incubations were with pH 5 tRNA methylase in a total volume of 0.3 ml with 200 μg of tRNA for 60 min. The pH 5 enzymes were recombined with their homologous high and low molecular weight fractions in the original proportions. Protein concentrations in the assays were: mouse liver pH 5 enzyme, 100 μg; mouse liver high molecular weight fraction, 300 μg; Ehrlich ascites cells pH 5 enzyme, 50 μg; Ehrlich ascites cells high molecular weight fraction, 100 μg.

* Values are in picomoles [^{14}C]methyl incorporated.

TABLE 5. *Effect of the inhibitor components from adult rabbit liver on the tRNA methylases of Novikoff hepatoma, Morris hepatoma, and Ehrlich ascites cells*

	Novikoff hepatoma		Morris hepatoma		Ehrlich ascites cells	
	Incorporation	% inhibition	Incorporation	% inhibition	Incorporation	% inhibition
Control	259.4	...	48.1	...	91.3	...
+ high MW fraction	26.2	90	14.6	70	20.9	77
+ low MW fraction	295.3	0	47.5	0	100.0	0

Assays were done with high-speed supernatant extracts of the tumor tissues in a total volume of 0.3 ml with 200 μg of tRNA for 60 min. Protein concentrations in the assays were: Novikoff hepatoma extracts, 500 μg; Morris hepatoma extract, 1.4 mg; Ehrlich ascites cells, 100 μg; and rabbit liver high molecular weight inhibitor fraction, 700 μg.

methylase capacity of tumor and fetal tissues: a breakdown in the normal regulation of these enzymes by interference with the tRNA methylase inhibitor. This possibility draws support from the finding reported here that the high molecular weight inhibitor found in adult tissues is absent from fetal and tumor tissues.

Chaney *et al.* (6), in a preliminary report, have noted the existence of a dialysable inhibitor of the tRNA methylases in

normal rat liver and claim its absence in Walker-256 carcinoma. Since their data, as presented, are difficult to interpret—i.e., the specific tRNA methylase activity of dialyzed tumor extracts is significantly enhanced, while the activity of extracts of normal tissues is not—it is impossible to resolve the apparent discrepancy between their findings and those reported here.

The similarity of the enzyme activities and of their inhibitors in embryonic and tumor tissues is noteworthy. The tRNA methylases modify the structure of a cardinal component of the protein-synthesizing system. Similarities of some proteins, i.e., the fetal antigens (7), have been noted between embryonic and some tumor tissues.

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