TRANSFER RNA METHYLASE INHIBITORS IN NEOPLASTIC AND NORMAL RAT TISSUE

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SUMMARY: With acid precipitation and dialysis, we show the presence of tRNA methylase inhibitors in normal adult rat liver. These inhibitors are virtually absent from the cortex of the highly malignant Walker-256 carcinoma from the rat. The tRNA methylase inhibitors of the rat liver are slowly dialyzable. It is proposed that the differences in the rate of tRNA methylase activity of normal and neoplastic tissues is in large part due to the difference in inhibitor content of each tissue.

The rate of methylation of methyl poor E. coli tRNA by extracts of neoplastic cells is in general considerably elevated above that of normal cells (1). Two recent publications have described results relating to this disparity (2,3). Leboy ascribed the difference in the rates of methylase activity to a variation in the concentration of polyamines present in the tissue extracts. Polyamines are known to exist in tissues at concentrations which profoundly influence the in vitro tRNA methylase reaction (2,4). Kerr (3) recently demonstrated the presence of methylase inhibitors in adult liver and their absence in fetal liver - a result which explains the dissimilarity in the rates of methylase activity of adult tissues compared with fetal tissues (3,5). Kerr also showed that the inhibitor prepared from normal adult rat liver had the capacity to inhibit the tRNA methylases from the Novikoff tumor.

We report here that there is a marked reduction in kind and concentration of tRNA methylase inhibitors in a highly malignant breast carcinoma of rats compared with normal adult rat liver. By measuring the

rate of tRNA methylase activity in extracts from normal and malignant tissues individually and in combinations before and after dialysis, some of the variables are eliminated and the fundamental difference between the two types of tissues is revealed.

METHODS: a. Preparation of extracts. The liver from a tumor bearing animal and its Walker-256 carcinosarcoma (W-256) were removed without delay from an ether anesthetized, exanguinated Sprague Dawley rat. Each was immediately immersed in cold buffer, pH 8.0 containing 0.25 M sucrose, 0.01 M $MgCl_2$ and 0.01 M tris (SMT buffer). Tissue excised from the outermost rim of actively growing tumor was first minced then washed once in SMT buffer and homogenized in a Potter Elvehjem homogenizer in two volumes of the same buffer. The liver was similarly prepared. Each homogenate was centrifuged for 10 minutes at 0°C at 17,300 x g. After removing the surface lipid layer, the supernatant was centrifuged at 105,000 x g for 60 minutes at 2°C. Any remaining surface lipids were removed by aspiration. The 105,000 g supernatant was used as the source of crude enzyme. This fraction was also treated with 1 N acetic acid to a final pH of 5. Five ml of the acidified suspension were centrifuged for 10 minutes at 0°C at 12,300 x g. The supernatant was separated from the precipitate and neutralized by the addition of 0.1 N NaOH, this fraction served as the source of inhibitor and referred to as liver neutralized supernatant (LNS) or tumor neutralized supernatant (TNS) dependent on its source. The precipitated enzyme was suspended in 1 ml of a pH 8.2 buffer containing 10 mM tris, 2 mM EDTA and 5 mM dithiothreitol. Aliquots of each of the fractions were dialyzed for 4 hours against 1 liter of SMT buffer with two changes as described by Kaye and Leboy (6). Protein concentrations of all fractions were measured by the method of Lowry (7). All pre- and post-dialysis assays were carried out after the same time interval from isolation of the fractions since changes in tRNA methylase activity were produced by standing at 4°C. This was particularly true of the crude enzyme preparations.

b. Assay: The reaction mixture in a volume of 0.4 ml contained 25 μmoles of tris pH 9; 160 mμmoles of dithiothreitol; 50 μg E. coli K 12 submethylated tRNA; 5.6 nmoles of ^{14}C methyl-S-adenosylmethionine (SAM), and enzyme in SMT buffer. The reaction mixture was incubated at 37°C for 60 minutes then stopped by the addition of 0.2 ml of neutral 4.0 M hydroxylamine. After an additional 20 minutes at 37°C the reaction tubes were placed on ice. 100 μl aliquots were spotted in duplicate on Whatman #3 mm filter paper discs, hot air dried, and washed by the procedure of Manns and Novelli (8). Following the cold trichloracetic acid washes, one set of duplicate discs was washed in hot (90°C) 5% trichloracetic acid for 30 minutes while the other set was left in cold 5% trichloracetic acid. Both sets of discs were then processed in the same way through trichloracetic acid, alcohol and ether. The filter pads were dried and placed in vials with toluene scintillation fluid for counting. Controls without added E. coli tRNA and blanks without enzyme were routinely performed.

TABLE I: Effect of Dialysis and Standing on tRNA Methylase Specific Activity

Enzyme Studies	Initial	After standing 4 hours 0°	After dialyzing 4 hrs.
Liver Crude Supernatant	51*	71	53
Tumor " "	69	9	325
Liver Neutralized "	17	17	24
Tumor " "	27	22	35
Liver pH 5 Precipitate	148	107	130
Tumor pH 5 Precipitate	130	128	141

*pmoles of [^{14}C] methyl groups incorporated into exogenous tRNA per mg protein per 60 minutes, assayed as outlined in the text.

When E. coli tRNA is added to the reaction mixture, the radioactivity precipitated by cold trichloracetic acid includes the methyl groups transferred to protein as well as to tRNA. The specific activity for methylation of exogenous tRNA was determined immediately after preparation of the fractions and again either standing or dialyzing for 4 hours at 4°C. The methylase specific activities for exogenous tRNA are recorded in Table I.

RESULTS AND DISCUSSION: The changes in the specific activity of the tumor crude extract on standing and on dialysis are particularly noteworthy and may account for some of the variations reported in the literature regarding the comparative rates of tRNA methylase activity in malignant and normal adult tissues. Because of the relative stability of the acid precipitated enzymes and the neutralized supernatants, they were used in all subsequent experiments with the precipitate as the source of enzyme and the supernatant as the inhibitor source (3).

In those assays involving undialyzed preparations, we used fractions which had stood in the cold the same length of time as those which had been dialyzed in the cold. To the liver precipitate were added increasing but comparable amounts of either liver neutralized supernatant (LNS) or tumor neutralized supernatant (TNS). The tRNA methylase rates were determined in each instance and are shown in figure 1a. It is clear from figures 1a and b that LNS contains an inhibitor active against both the liver enzyme and against the tumor enzyme while TNS appears to stimulate both enzymes.

After dialysis of both the precipitated enzymes and their neutralized supernatant portions, they were again combined and assayed as before dialysis. The results shown in figures 2a and 2b clearly indicates that the inhibitor is dialyzed away and LNS like TNS becomes stimulatory to both enzymes.

By dialyzing all preparations as suggested by Kaye and Leboy (6) and resorting to acid precipitation as devised by Kerr (3), the changes due to

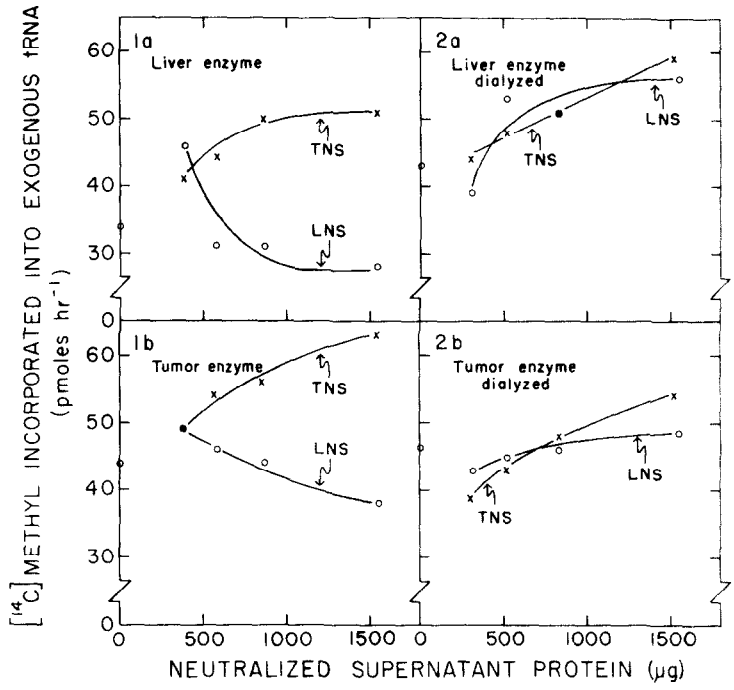

Figure 1. tRNA Methylase; Effect of Liver and Tumor Neutralized Supernatant.

The enzyme was precipitated at pH from liver, A, (320 µg) and from Tumor, B, (345 µg). In each case enzyme activity was determined alone and with increasing LNS or TNS (300 to 1500 µg). tRNA methylase activity was determined by the method outlined in the text.

Figure 2. tRNA Methylase; Effect of Dialyzed Liver and Tumor Neutralized Supernatants on the Dialyzed Enzyme.

Conditions as in Figure 1 except all fractions were dialyzed 4 hours against SMT buffer and assayed as outlined in the text.

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polyamines and the effects of standing have been largely eliminated. Ammonium acetate addition to the reaction mixture was excluded because, as previously reported by Kerr (3), ammonium ion interfered with the effect of the inhibitor, and as both Kerr (3) and Leboy (2) reported, direct

addition of ammonium acetate to the enzyme stimulated methylase activity. By the addition of the neutralized supernatant preparations containing both enzyme and inhibitor to the acid precipitated enzymes, the difference between the inhibitors in liver and tumor became clearly evident. The liver contained considerable inhibitor whereas the most actively growing part of the neoplasm was practically devoid of inhibitor.

The inhibitor could be very slowly removed from the LNS by dialysis against distilled water. Five ml of LNS were dialyzed for 18 hours. The dialysate was lyophilized and redissolved in 5 ml of distilled water. Addition of aliquots of the reconstituted dialysate to the liver enzyme produced inhibition as shown in Table 2.

TABLE II: Effect of Dialyzed Inhibitor on tRNA Methylase

System	Activity
1. Dialyzed Liver pH 5 Precipitate	192*
2. (1) + 100 µL dialyzed substance	100
3. (1) + 200 µL dialyzed substance	97

*pmoles of [^{14}C] methyl groups incorporated into exogenous tRNA per mg protein per 60 minutes, assayed as outlined in text.

In conclusion we propose that coincident with the malignant transformation of cells there is not only an increase in the rate of tRNA methylase activity (2) but this increase may be due to different and, diminished levels of tRNA methylase inhibitors in neoplastic cells.

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