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Transfer RNA Methyltransferase and Glycine *N*-Methyltransferase Activity during *Rana pipiens* Development

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Changes in the activity of the tRNA methyltransferases have been found in all differentiating systems studied. Activity was examined in extracts of *Rana pipiens* embryos and in larval and adult liver by *in vitro* assay using *S*-adenosyl-L-[*methyl*-^{14}C]methionine as the methyl donor. Specific activities of tRNA methyltransferases decreased, beginning with the time of feeding, when using high concentrations of the crude liver enzyme. A new methyltransferase activity, glycine *N*-methyltransferase, appeared at the time of feeding. Apparently, the glycine methyltransferase is active before the onset of any of the characteristic metamorphic changes of other liver enzymes. Using partially purified enzyme from adult liver, the K_m of glycine methyltransferase for *S*-adenosylmethionine is 0.3 m*M* and the K_i for *S*-adenosylhomocysteine, a competitive inhibitor, is 0.08 m*M*.

INTRODUCTION

Many biological systems undergoing changes in regulation have been studied with respect to changes in activity of tRNA methyltransferases (summarized in Kerr, 1974; Law *et al.*, 1976, 1977). The variable additions of methyl groups to the bases of tRNA at the macromolecular level, as well as other modifications of tRNA, will change the structural conformation of the molecules and may change the fit to ribosomes (Fittler and Hall, 1966; Thiebe and Zachau, 1968; Gefter and Russell, 1969), the interaction with DNA (Singer *et al.*, 1972), codon recognition (Capra and Peterkofsky, 1968; Smith, 1975) or aminoacylation (Shugart *et al.*, 1968). Regions of tRNA which could be altered structurally and have functional significance have been described by Quigley and Rich (1976). The possibilities of fine control by changing tRNAs after transcription exists because the methyltransferases are specific for each organism species, tissue type, base, and base sequence. Differences in levels of methylation within various specific tissues over the developmental period of the organism may be in part due to competing enzyme systems which generally appear during early post-embryonic stages and increase to adult stage (Kerr, 1972, 1974). The actual systems vary from tissue to tissue, but they have in common (1) competition for substrate, *S*-adenosylmethionine, and (2) differential sensitivity to inhibition by the end-product *S*-adenosylhomocysteine.

Metamorphosis in amphibians is a unique secondary time of developmental change which is dependent on the hormone thyroxine (Kollros, 1961). In a preliminary study, Pillinger *et al.* (1971) found changes in tRNA methyltransferase activity in liver and tail tissue in thyroxine-stimulated *Rana catesbeiana* larvae. Since many liver enzymes are newly synthesized or change from larval to adult forms at the time of metamorphosis, the appearance of a new methyltransferase activity might be dependent on the hormone thyroxine.

Data presented will show that tRNA methyltransferase activity from liver does not change dramatically at metamorphosis, but that a new methyltransferase activity, glycine methyltransferase, appears at the

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time of feeding in *R. pipiens*. The appearance or activation of glycine methyltransferase does not depend on the hormone thyroxine, because livers from hypophysectomized animals have glycine methyltransferase activity.

MATERIALS AND METHODS

Chemicals. *S*-Adenosyl-L-[*methyl*-^{14}C]-methionine (51.8 mCi/mmole) was purchased from International Chemical and Nuclear Corporation. *Escherichia coli* tRNA was bought from General Biochemicals. Hydroxylapatite was the product of Bio-Rad.

Animals. Sexually mature *R. pipiens* were obtained from C. Mumley Frog Farm, Alburg, Vermont. Some sexually mature females and some stages of embryos and larvae were purchased from The Amphibian Facility, University of Michigan, Ann Arbor. Females were induced to ovulate by intraperitoneal injection of from 2 to 5 *R. pipiens* pituitaries 24 to 48 hr before the time of fertilization. Males were sacrificed and the testes crushed to provide viable spermatozoa. Any defective embryos were discarded.

All premetamorphic animals were raised at 21–25°C in aerated tap water. Adults were used immediately on arrival or kept in tap water at 4°C until they were used.

Some frozen adult *R. pipiens* livers were purchased from Pel-Freez Biologicals, Inc., Rogers, Arkansas.

Hypophysectomy. Embryos of stages 17–18 were hypophysectomized using glass needles according to established methods (Rugh, 1962). These animals were raised separately from normal animals and used only after all of the siblings had gone through metamorphosis (from 8 to 12 months after fertilization).

Preparation of enzyme extracts. Animals were sacrificed by decapitation after slowing in water at 4°C or in anesthetic, ethyl-*m*-aminobenzoate methanesulfonic acid (0.01%). Tissues were used immediately for tests for tRNA methyltransferase activity, but some were frozen at −20°C for tests for glycine methyltransferase activity.

Liver or embryos were minced and homogenized in 4 vol of 0.01 *M* Tris–HCl, pH 7.4, 0.01 *M* NaCl, and 0.0015 *M* $MgCl_2$ using a motor-driven Teflon–glass tissue grinder. This homogenate was centrifuged at 15,000*g* for 10 min and the resulting supernatant was centrifuged at 105,000*g* for 60 min. The high-speed supernatant was used directly to measure the tRNA methyltransferase activity. The tRNA methyltransferase enzymes were removed by precipitation with 1.0 *M* acetic acid to pH 5.0 and subsequent centrifugation at 10,000*g* for 10 min; glycine methyltransferase was left in the supernatant. The supernatant was reneutralized with 1.0 *M* Na_2CO_3. The preparation was directly desalted on a Sephadex G-25 column to remove glycine or was concentrated with the addition of 4.0 g $(NH_4)_2SO_4$ per 10 ml of solution before desalting. The high-molecular-weight column eluate was used for glycine methyltransferase activity assays.

Partial purification of glycine methyltransferase. Twenty to forty grams of adult liver was prepared as above. The concentrated pH 5.0 supernatant was dissolved in hydroxylapatite column buffer, 0.01 *M* sodium phosphate, pH 7.1, 0.01 *M* Tris–HCl, and 0.001 *M* dithiothreitol and desalted on a Sephadex G-25 column. The high-molecular-weight eluate was dialyzed at 4°C for 24 hr against three changes of column buffer. At the same time a 2.5 × 9.0-cm hydroxylapatite column was poured and washed at 4°C with column buffer. A 300-ml linear gradient of 0.01 to 0.1 *M* sodium phosphate in column buffer was used to elute the proteins. Column fractions were analyzed for glycine methyltransferase activity and the peak fractions were pooled and used for kinetic experiments.

Assay of tRNA methyltransferases. The standard assay mixture contained 10 μmole of Tris–HCl, pH 8.2, 1 μmole of $MgCl_2$, 0.2

μmole of dithiothreitol, 10 μg of *E. coli* B tRNA, 2 nmole (0.1 μCi) of *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and varying amounts of enzyme extract in a total volume of 0.2 ml. Control assays were done without tRNA. After incubation at 27°C for 30 min the reaction was terminated by the addition of 0.2 ml of 10% TCA. Precipitates were collected on Whatman GF/C filters which had been pretreated with 0.1 *M* EDTA; the filters were next washed with 5% TCA. The filters were dried under an infrared lamp and were counted in a Beckman 8100 liquid scintillation counter with a counting efficiency of 90–95%.

Assay of glycine methyltransferase. The standard assay mixture contained 10 μmole of Tris-HCl, pH 8.6, 0.2 μmole of dithiothreitol, 1 μmole of glycine, 2 nmole (0.1 μCi) of *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and varying amounts of enzyme extract in a total volume of 0.2 ml. Control assays were done without glycine. After incubation at 27°C for 30 min for enzyme activity assays, 5 μmole of adenosine was added as a carrier and the assays were precipitated at 4°C with 50 μl of 20% phosphotungstic acid. Distilled water at 4°C was added to a final volume of 1 ml. The unreacted *S*-adenosylmethionine and the precipitated protein were removed by centrifugation at 30,000*g* for 20 min. An aliquot of 100 μl of the supernatant containing the labeled product, sarcosine, was added to 7 ml of Tritosol (Fricke, 1975) and counted in a Beckman 8100 scintillation counter with a counting efficiency of 70–74%. Some of the conditions were varied for kinetic assays as detailed in Fig. 2.

In order to check for the authenticity of the methylated product, sarcosine, two-dimensional thin-layer chromatography of the supernatant described above and of glycine and sarcosine standards was done (Kerr, 1972).

Assay of protein. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

RESULTS

tRNA Methyltransferase Activity

tRNA methyltransferase activity measured by assays of incorporation of $^{14}CH_3$ groups of *S*-adenosylmethionine into a saturating level of substrate, *E. coli* B tRNA, is high in prefeeding *R. pipiens* embryos and in hypophysectomized larval liver. The activity is lower in normal-feeding-stage larvae and in adult liver (Fig. 1). Liver extracts from normal-feeding-stage larvae of Stage I (Taylor and Kollros, 1946) through metamorphosis and from adults have decreasing activity with increasingly high levels of protein in assays.

Glycine Methyltransferase Activity

Activation of the enzyme system, glycine methyltransferase, occurred at the time of feeding (Stage I) in *R. pipiens* larvae (Table 1). Glycine methyltransferase activity was measured by the assay of incorporation of $^{14}CH_3$ groups of *S*-adenosylmethionine into a saturating level of glycine. Generally the appearance and the level of glycine methyltransferase activity vary inversely with the level of tRNA methyltransferase activity.

Two-dimensional thin-layer chromatography of the reaction verified that the radioactivity above the background value was present coincident with the sarcosine standard.

Characterization of Partially Purified Glycine Methyltransferase

The *R. pipiens* glycine methyltransferase requires Mg^{2+}, a sulfhydryl reagent, and is maximally active under the assay conditions used between pH 8.0 and 8.8. The crude or relatively pure enzyme is not stable in solution when stored at 4 or at −20°C. Antibody to rabbit liver glycine methyltransferase (Heady and Kerr, 1975) did not cross-react with the enzyme from *R. pipiens* liver. Glycine methyltransferase displays Michaelis-Menten kinetics. When the concentration of *S*-adenosylmethionine

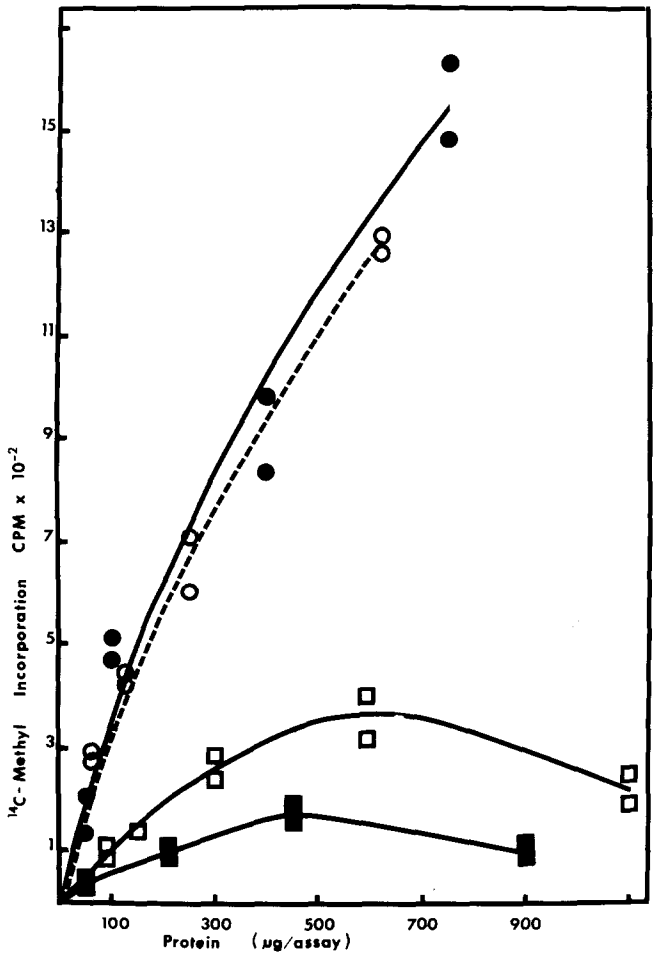

FIG. 1. tRNA methyltransferase activity during development in *Rana pipiens*. The incorporation of $^{14}CH_3$ groups from *S*-adenosylmethionine into *Escherichia coli* B tRNA was measured as described under Materials and Methods. Control assay values have been subtracted from all experimental points. The points are representative assays for embryos (●——●), larval stage IX–X liver (□——□), hypophysectomized larval liver (○- - -○), and adult liver (■——■).

is varied and glycine is saturating, the Michaelis constant (K_m) for *S*-adenosylmethionine is 0.3 m*M*. The enzyme is competitively inhibited by the product *S*-adenosylhomocysteine; the K_i value is 0.08 m*M* (Fig. 2).

When the concentration of glycine is varied and *S*-adenosylmethionine is saturating, the Michaelis constant (K_m) for glycine is 0.41 m*M*.

DISCUSSION

The activity of tRNA methyltransferases from embryonic *R. pipiens* is high compared with that of normal larval and adult liver. This pattern follows other systems which have been tested (Kerr, 1974). The glycine methyltransferase enzyme system of *R. pipiens* is comparable to that of mammals with respect to time of its appearance, at larval–fetal stages (Heady and Kerr, 1975), and the conditions of enzyme activity and kinetics (Heady and Kerr, 1973). The Michaelis and inhibitor constants of rabbit liver glycine methyltransferase for *S*-adenosylmethionine are 0.1 and 0.035 m*M* (with *S*-adenosylhomocysteine), respectively (Heady and Kerr, 1973). However, the

TABLE 1

GLYCINE METHYLTRANSFERASE ACTIVITY AND tRNA METHYLTRANSFERASE ACTIVITY IN *R. pipiens* LIVER[a]

Tissue	Glycine methyltransferase (nmol/mg/30 min)	tRNA Methyltransferase (nmol/mg/30 min)
Embryos[b]	Not detected	0.029
Larva[c]		
I-II	20.4	0.031
VI-VIII	19.2	0.007
IX-XIII	25.4	0.013
Adult	45.5	0.008
Metamorphic climax		
XX-XXIV[c]	10.2[d]	0.005
Hypophysectomized[e]	8.6	0.021

[a] Values are averages of from three to six separate determinations. Control assay values have been subtracted from all experimental points.

[b] Total embryos of Stages 21-25.

[c] Taylor-Kollros stages.

[d] Repeated tests were low. This could indicate complex enzyme interactions during metamorphic climax.

[e] From 8 to 12 months of age, with no more than Stage VII leg.

mammalian enzyme is stable in solution at 4°C for several weeks, but the frog enzyme is not stable in solution. Antibody to rabbit glycine methyltransferase does not cross-react with the frog liver enzyme.

The anuran was chosen for this developmental study, because metamorphosis is a time of new synthesis in the liver and this synthesis is controlled by the hormone thyroxine. Although many liver enzymes change during metamorphosis (Cohen, 1970), glycine methyltransferase activity does not appear to be controlled by thyroxine. Glycine methyltransferase activity is measurable before the premetamorphic stages of development (during Stages I-VIII) when thyroxine levels are very low (Kollros, 1961; Etkin, 1964) and in hypophysectomized animals where thyroxine is absent.

A change in relative amounts of two major leucyl tRNAs has been reported in *R.*

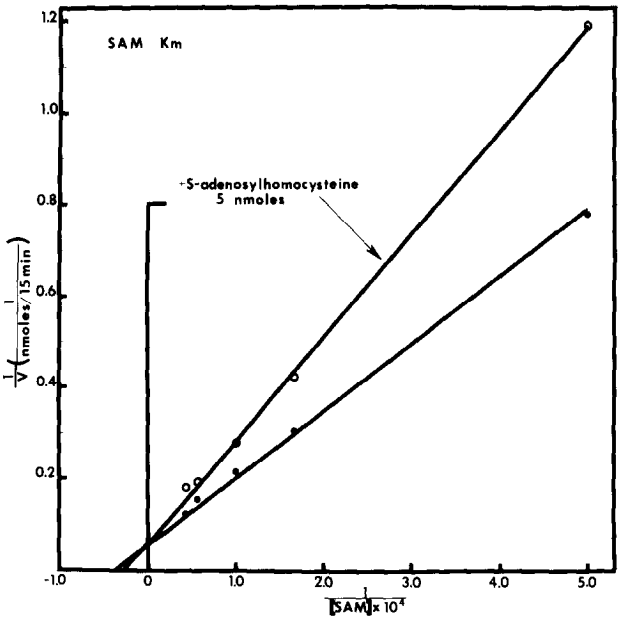

FIG. 2. Determination of the kinetic constant of *S*-adenosylmethionine. Values represent the averages of quadruplicate assays. Assays had 13 μg of glycine methyltransferase and were incubated for 15 min at 27°C. Assays contained 2 μmole of glycine and varying amounts of *S*-adenosylmethionine in the absence of *S*-adenosylhomocysteine (●——●) and in the presence of 5 nmole of *S*-adenosylhomocysteine (○——○). Control assay values have been subtracted from all experimental points. The lines were drawn to fit the points with the help of a computer program for a least-squares plot.

catesbeiana liver during spontaneous and triiodothyronine-induced metamorphosis (Tongue *et al.,* 1969), a time of new protein synthesis. The new conditions including altered relative amounts of different amino acids require correspondingly altered relative amounts of some species of tRNAs (Smith, 1975; Garel, 1976). A species of tRNA could be limiting until a certain point in development when this species might be synthesized preferentially or in some cases might be differentially modified postsynthetically (Smith, 1975). The differential modification might include methylation of tRNAs by tRNA methyltransferases competitively controlled by enzyme systems such as glycine methyltransferase.

Pillinger *et al.* (1971) reported that during thyroxine-induced metamorphosis the activity of tRNA methyltransferases changed in specific patterns in tail tissue and in liver and that possibly a hormone-controlled inhibitor system was involved. The decrease in activity of tRNA methyltransferase was transitory. It is not known if a competing enzyme was involved. However, there are differences in activities of tRNA methyltransferases between hypophysectomized and normal larvae when high protein concentrations are used (Fig. 1 and Table 1). Although the crude enzyme preparation (105,000*g* supernatant) contained both enzymes, each might be compartmentalized within living cells.

There is some preliminary evidence that glycine methyltransferase might act as a competitive enzyme system in larval and adult frog liver. First, as protein concentration increases in tRNA methyltransferase assays, using crude 105,000*g* supernatant, activity is depressed (Fig. 1). Second, there is a general correlation of the presence of glycine methyltransferase and this depression. Third, when the pH 5.0 precipitated protein from all normal larval and adult stages is used as the tRNA methyltransferase enzyme source there is a linear increase in activity as shown for the crude supernatant of the embryos (Fig. 1) (Heady, unpublished; Kerr, 1974). In this case the glycine methyltransferase activity remains in the pH 5.0 supernatant and is removed from the reaction.

Changes in the tRNA population of certain tissues due to hormone stimulation might also alter the protein synthesis quantitatively. In a tRNA-dependent *in vitro* protein-synthesizing system almost twice the ovalbumin is synthesized when the tRNA extracted from estrogen-stimulated chick oviducts or laying-hen oviducts is used compared with tRNA extracted from withdrawn chick oviducts (Sharma *et al.,* 1975, 1976).

Glycine methyltransferase makes up 2.5% of the soluble liver protein in the rabbit (Heady and Kerr, 1975) and although the percentage of glycine methyltransferase in frog liver is not known, it is significant. This enzyme would seem to be an important part of vertebrate digestive glands [glycine methyltransferase is also present in mammalian pancreas (Kerr, 1972)]. The presence of the enzyme system in adult crustacean digestive glands, *Callianassa gigas* and *Homarus americanus* (Heady, unpublished), further lends support to the possible significance of this enzyme for control of the critical activity of tRNA methyltransferases during development and possibly for indirect control of other enzymes using *S*-adenosylmethionine or *S*-adenosylhomocysteine as substrate (Kerr and Heady, 1974).

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