Hormonal Effect on Transfer Ribonucleic Acid Methylases and on Serine Transfer Ribonucleic Acid*

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ABSTRACT: Ovariectomy produces a 40% diminution in the capacity of extracts of pig uteri to introduce methyl groups into heterologous transfer ribonucleic acid (tRNA). The $tRNA^{Ser}$ from normal uteri elutes at higher salt concentration on MAK column than the one from an ovariectomized uterus. Administration of physiological doses of estradiol to the ovariectomized animals restored the methylase capacity and $tRNA^{Ser}$ elution profile to that of normal uteri. Ovariectomy or the administration of estradiol to ovariectomized animals had no effect on tRNA methylases nor on the elution profile of $tRNA^{Ser}$ of the liver.

The changes observed in tRNA methylases in uteri appear to be due to the presence of different levels of inhibitor(s) of tRNA methylases.

tRNA methylases achieve a structural modification of tRNA. The reaction involves transfer of methyl groups from *S*-adenosyl-L-methionine to accepting sites in tRNA (Fleissner and Borek, 1962). The enzymes are invariably constant in homogeneous biological systems in the same phase of growth. Thus enzymes in bacteria in logarithmic growth phase or of adult mammalian tissues will invariably methylate sites *in vivo* or *in vitro* characteristic of the organism. However, in organisms or tissues undergoing some process of differentiation there are profound alterations both qualitative and quantitative in the enzymes. Changes in tRNA methylases have been observed in insects during metamorphosis (Baliga *et al.*, 1965), on phage infection (Wainfan *et al.*, 1965), in embryonic and neonatal tissues (Hancock *et al.*, 1967), in lens tissue during differentiation (Kerr and Dische, 1968), in the slime mold during morphogenesis (Pillinger and Borek, 1969), and in a variety of tumor tissues (Borek, 1969). The altered patterns of methylation observed *in vitro* have been confirmed *in vivo* (Bergquist and Mathews, 1962; Viale *et al.*, 1967; Craddock, 1969). The control mechanisms governing the changes in the enzymes are not known. However, inhibitors of tRNA methylases have been observed in induced lysogenic organism (Wainfan *et al.*, 1966), in adult organs of rabbit and rat (Kerr, 1970) and in the differentiating slime mold *Dictyostelium discoideum* (Sharma and Borek, 1970).

Recent experiments (Lipshitz-Wiesner *et al.*, 1970) showed that ovariectomy invariably diminishes by about one-half the capacity of extracts of rat uteri to introduce methyl groups *in vitro* into heterologous tRNA. These experiments were performed on the pooled uteri of 10–12 rats and the studies included 6 such groups of normal and ovariectomized animals. The reduction in tRNA methylase capacity was not uniform for various base-specific enzymes. Administration of estradiol to the ovariectomized animals restored the methylase capacity to normal. These findings prompted the suggestion that the alteration in the methylation pattern by the hormone may also produce differences among the tRNA populations.

Due to the limited amounts of uterine tissue from rats this possibility could not be explored. We have, therefore, transferred our studies to the pig uteri. Now we report alterations in tRNA methylases, their inhibitor(s), and a change in the population of serine tRNA as a consequence of ovariectomy in the target organ. All of these alterations are restricted to the target organ and they are all reversed by the administration of physiological doses of estradiol.

Materials

Five pigs weighing 12–15 kg were ovariectomized by Dr. Warren Frost of this institution and were kept on a farm on an unrestricted diet. All subsequent procedures were done 3 months after the operation. 17β-Estradiol (Calbiochem) was injected intramuscularly (40 μg/kg body weight) for 3 days to the ovariectomized animals. The animals were sacrificed under Nembutal anesthesia and the uterus and liver were removed immediately and were chilled.

Methylated albumin was prepared by the method of Mandell and Hershey (1960) using crystalline bovine serum albumin fraction V from Nutritional Biochemicals Corp. L-Serine-^{14}C (sp act. 137 Ci/mole) and -*t* (sp act. 3790 Ci/mole) was purchased from New England Nuclear. Other L-amino acids both ^{14}C and ^{3}H were obtained from Amersham Searle. *S*-Adenosyl-L-methionine (methyl-^{14}C, sp act. 46.5 Ci/mole) was a product of International Chemical and Nuclear Corp. *E. coli* B tRNA was obtained from General Biochemicals Corp.

Methods

Preparation of Enzyme Extracts for Methylase Assay. The chilled tissues were minced and were homogenized in 4 volumes of 0.25 M sucrose, 0.02 M Tris-HCl, pH 8.2, 0.01 M $MgCl_2$, and 0.005 M mercaptoethanol in a cold Sorval Omni-Mixer for 3 min at full speed. The homogenate was

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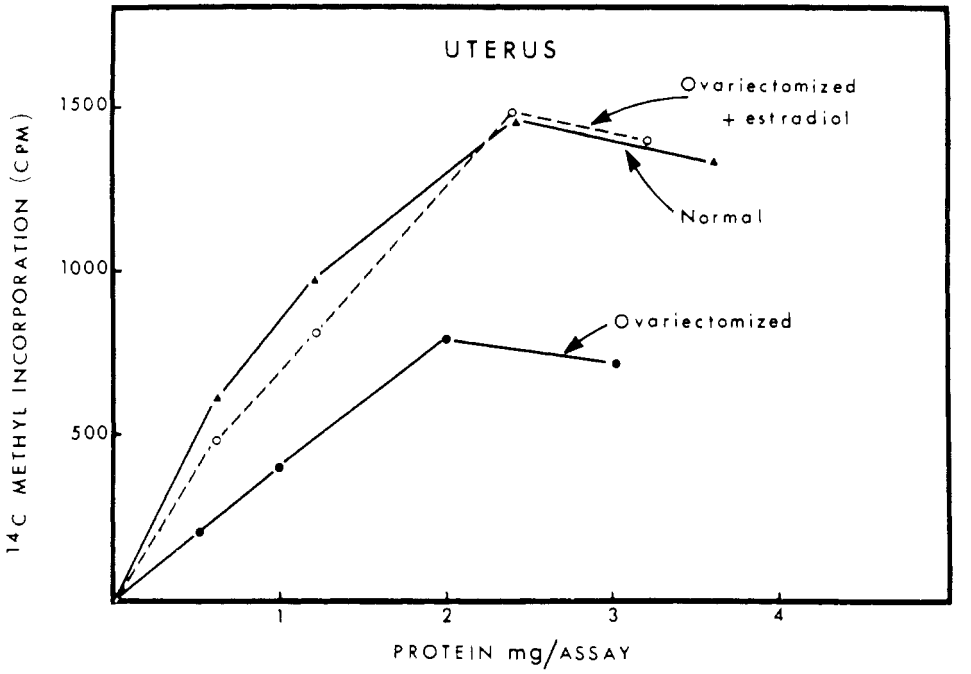

FIGURE 1: Methylation of E. coli B tRNA by 100,000*g* supernatant of pig uteri of normal, ovariectomized, and ovariectomized estradiol treated. Assay mixture contained 50 μg of *E. coli* B tRNA. The enzymes of six normal pigs were examined. The variation of incorporation at saturation enzyme levels was ±10%. The tRNA methylases of four ovariectomized pig uteri were studied. The variation among these was also ±10%. One ovariectomized pig was administered estradiol.

centrifuged at 12,000*g* for 10 min and then at 100,000*g* for 1 hr. The high-speed supernatant was used as source of tRNA-methylating enzymes. The protein content of the solution was measured by the method of Lowry *et al.* (1951).

Assay of tRNA Methylase. The enzyme activity was assayed by measuring the incorporation of methyl-^{14}C groups of *S*-adenosylmethionine into *Escherichia coli* B tRNA. The reaction mixture contained: Tris-HCl, pH 8.2 (100 μmoles), $MgCl_2$ (10 μmoles), mercaptoethanol (10 μmoles), *E. coli* B tRNA (50 μg), *S*-adenosyl-L-methionine (methyl-^{14}C, 0.2 μCi, sp act. 46.5 Ci/mole), and enzyme in a total volume of 1 ml. The control tubes received no tRNA. After incubation at 37° for 30 min the reaction was terminated by the addition of 2 ml of chilled absolute ethanol. To each tube 100 μg of yeast RNA was added as carrier. The tubes were chilled in ice and after 20–30 min the precipitates were washed 3 times with 67% ethanol containing 0.1 M sodium acetate. The pellets were dissolved in 1 ml of 0.2 M NH_4OH, which was placed in planchets, dried, and counted in a gas-flow counter (Nuclear-Chicago). The efficiency of counting was about 25%.

Preparation of tRNA. Transfer RNA was prepared by homogenizing the minced tissue in Sorval Omni-Mixer with 5 volumes of 1.0 M NaCl–0.005 M EDTA in 0.1 M Tris-HCl, pH 7.5, and an equal volume of water-saturated phenol for 3 min (Brunngraber, 1962). The aqueous layer was separated from the phenol layer by centrifugation, and was again extracted with an equal volume of phenol. The RNA was precipitated from the aqueous layer by the addition of 2.5 volumes of 95% ethanol containing 2% potassium acetate. After standing at −20° overnight, the precipitate was collected by centrifugation and was drained free of ethanol. The precipitated RNA was extracted with 1 M NaCl in 0.1 M Tris-HCl, pH 7.8, in the cold and was again precipitated with ethanol. It was dissolved in 10 ml of 0.01 M $MgCl_2$–0.1 M Tris-HCl, pH 7.5, and 200 μg of RNase-free DNase (Worthington) was added. The solution was incubated at 37° for 30 min. The reaction mixture was deproteinized with an equal volume of water-saturated phenol and tRNA was precipitated from the aqueous layer by 3 volumes of ethanol. Stripping off endogenous amino acid was done by incubating in 10 ml, 1.8 M Tris-HCl, pH 8.0, at 37° for 90 min (Sarin and Zamecnik, 1964).

Preparation of Aminoacyl-tRNA. The tRNAs were acylated in a reaction mixture containing: Tris-HCl (pH 7.4, 100 μmoles), $MgCl_2$ (5 μmoles), mercaptoethanol (5 μmoles), KCl (10 μmoles), ATP (pH 7.4, 5 μmoles), CTP (pH 7.4, 5 μmoles), 1 μCi of ^{14}C-labeled amino acid or 2–10 μCi of ^{3}H-labeled amino acid, enzyme preparation (2 mg), and 0.2 mg of tRNA in a total volume of 1 ml. After 10-min incubation at 37° the mixture was deproteinized with water-saturated phenol. The RNA was precipitated from the aqueous layer by 2.5 volumes of ethanol. A crude preparation of aminoacyl-tRNA synthetase prepared from rat liver (Axel *et al.*, 1967) was used for charging tRNAs from normal as well as ovariectomized animals.

Methylated Albumin Kieselguhr (MAK) Column Chromatography. To the solution of aminoacylated tRNAs, 2 mg of *E. coli* B tRNA was added as carrier and the volume was made up to 5 ml in 0.3 M NaCl and 0.05 M sodium phosphate buffer, pH 6.7, which was then applied to the MAK column (1.5 × 25 cm). The column was washed with the starting buffer until no ultraviolet-absorbing material was eluted. A linear gradient of 0.3–0.6 M NaCl or 0.3–0.7 M NaCl in 0.05 M phosphate buffer, pH 6.7, was applied. The flow rate was adjusted to 1 ml/min and 2-ml fractions were collected.

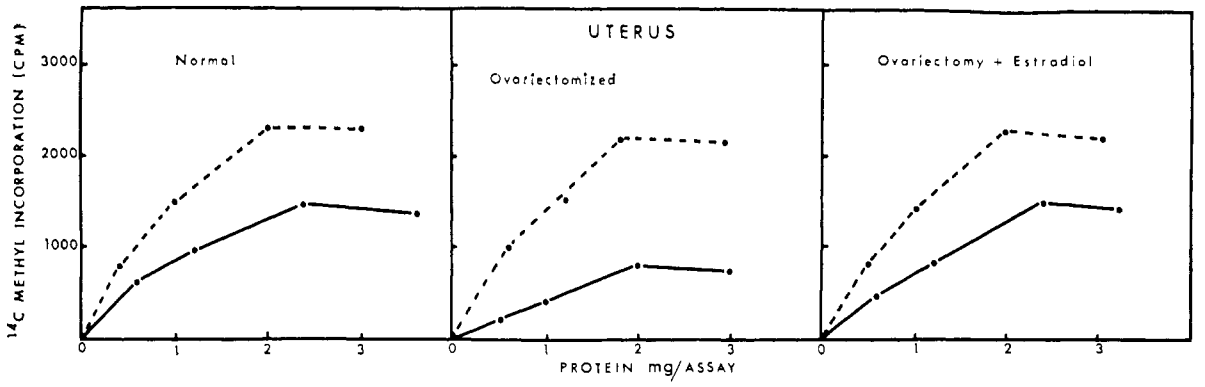

FIGURE 2: Effect of pH 5.0 precipitation of 100,000*g* supernatant on methylase activity. The 100,000*g* supernatant was adjusted to pH 5.0 with 0.1 N acetic acid. The precipitate was centrifuged. The methylase activity of pH 5.0 precipitate was determined by dissolving the precipitate in 0.05 M Tris-HCl, pH 8.2, and making the volume agree with the original volume of the 100,000*g* supernatant: (●—●) 100,000*g* supernatant; (●·····●) pH 5.0 precipitate.

Determination of Radioactivity. To each tube from the MAK column 200 μg of calf thymus DNA (Nutritional Biochemicals Corp.) was added followed by an equal volume of 10% trichloroacetic acid. The precipitated RNA was collected on glass fiber filter (Whatman GF/C) in a Millipore sampling manifold and was washed three times with 5% ice-cold trichloroacetic acid. The filters were dried for 1 hr at 80°. Scintillation fluid (8 ml, 0.4%, 2,5-diphenyloxazole, 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene) was added to each vial and radioactivity was counted in a Nuclear-Chicago scintillation counter Mark 1. For double-labeled samples with ^{14}C and ^{3}H the efficiency of counting for ^{14}C was 64% and that of ^{3}H 21%. There was less than 5% spilling of ^{14}C radioactivity into the ^{3}H channel.

To make certain that the alterations found in the tRNA^{Ser} profiles were not the spurious result of some contaminant in the labeled amino acid, the amino acids with the different labels were interchanged between tRNAs from different sources.

Results

Methylase Activity in the Organs of Normal, Ovariectomized, and Estradiol-Treated Animals. The methylase activity was assayed under optimum conditions and results are expressed as saturation capacity, *i.e.*, further increments of enzyme to the incubation mixture produced no further incorporation of methyl groups. Among the uteri of six different normal animals the variation in methylase capacity was within ±10%. This accords with the characteristics of mixtures of tRNA methylases extracted from other sources of stable biological systems. Among the uteri of four ovariectomized pigs the tRNA methylase capacities also varied only ±10% but crude tRNA methylase from extracts of uteri of ovariectomized pigs had about 60% the total capacity to introduce methyl groups into a heterologous substrate compared with the normal uterus (Figure 1). Following the administration of estradiol to an ovariectomized animal, the methylase capacity was restored to that of the uterus of normal animals. While for reasons of economy only one ovariectomized pig was administered estradiol, earlier experiments with scores of ovariectomized rat uteri showed a consistent return of the methylase capacity of the uteri to that of intact animals. (Lipshitz-Wiesner *et al.*, 1970).

The decrease in methylase capacity in the ovariectomized uteri could be due either to the destruction of some of the tRNA methylases or to the inhibition of some of the enzymes. To resolve these two possibilities use was made of the observation of Kerr (1970) that precipitation at pH 5.0 of the mixture of crude enzymes removes an inhibitor from the precipitated enzymes of mammalian tissues.

High-speed supernatants from normal, ovariectomized, and estradiol-treated animals were brought to pH 5.0 with

TABLE 1: Effect of Preincubation of tRNA with Inhibitor on Methylase Activity.[a]

Experiment	Methyl-^{14}C Incorporated		Reduction in Activity (%)
	cpm	μμmoles	
tRNA, control preincubated with water	520	20.8	(0)
tRNA, preincubated with inhibitor	305	12.2	41.3
tRNA, preincubated with inhibitor and then heat inactivated	490	19.6	5.7

[a] *E. coli* B tRNA (100 μg) was incubated with water or 2.5 mg of pH 5.0 supernatant from ovariectomized pig uteri (adjusted to pH 8.2) in Tris-HCl (pH 8.2, 100 μmoles), $MgCl_2$ (10 μmoles), mercaptoethanol (10 μmoles), in a total volume of 0.5 ml, for 30 min at 37°. The inhibitor was inactivated by immersing the tubes in boiling water for 5 min. The tubes were chilled in ice, 2 mg of enzyme (pH 5.0 precipitate, dissolved in 0.05 M Tris-HCl, pH 8.2), 0.2 μCi of *S*-adenosyl-L-methionine, and water to make a final volume of 1 ml, was added and the methylase activity was assayed by incubating at 37° for 30 min. Identical results were obtained with normal and ovariectomized pigs treated with estradiol.

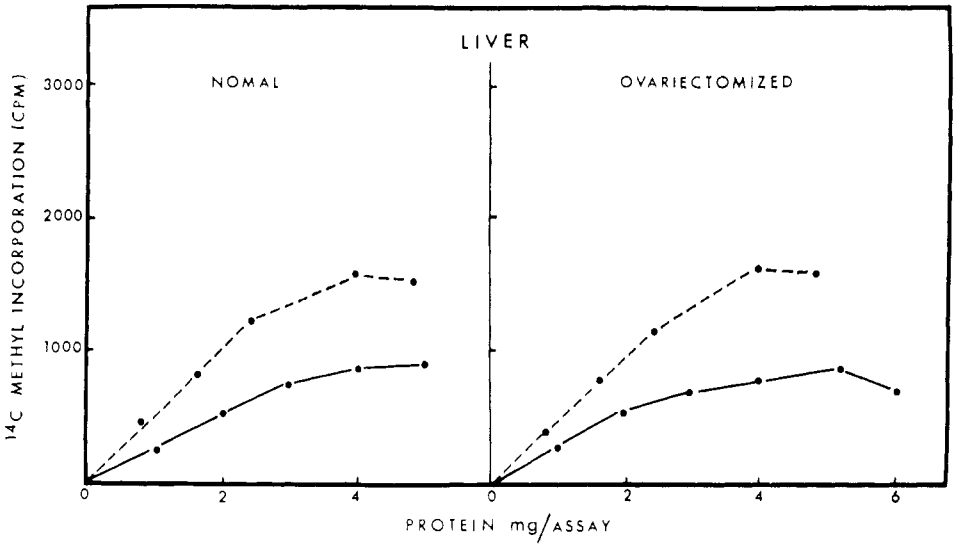

FIGURE 3: Methylase activity of 100,000*g* supernatant and pH 5.0 precipitate of normal and ovariectomized pig liver: (●——●) 100,000*g* supernatant; (●-----●) pH 5.0 precipitate.

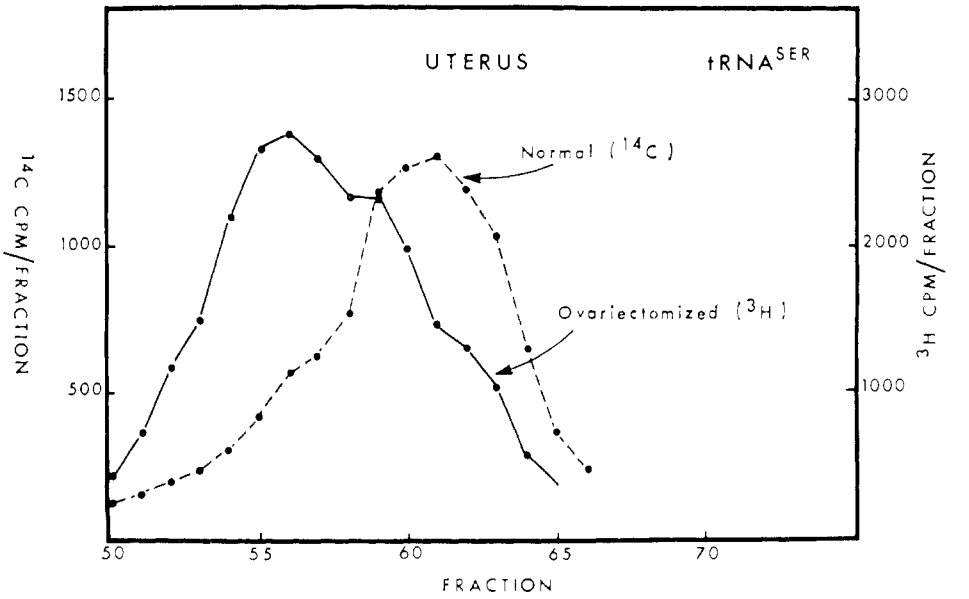

FIGURE 4: Methylated albumin kieselguhr (MAK) column chromatography of tRNA^{Ser} from normal and ovariectomized pig uteri. Hyflo Super Cel (20 g) (Fisher Scientific) was suspended in 0.3 M NaCl in 0.05 sodium phosphate buffer, pH 6.8, and was boiledfor 2 min to expel air. After cooling in ice 50 mg of methylated albumin dissolved in 5 ml of water was slowly added to Hyflo Super Cel under stirring. The stirring was continued for 15 min more. MAK was packed in a 1 × 25 cm column under air pressure and was washed with 0.3 M NaCl in phosphate buffer, pH 6.8, till no ultraviolet-absorbing material was eluted. The solution of RNA to be chromatographed was applied to the column and washing was continued with starting buffer until an optical density monitor maintained a steady base line. A linear gradient of 100 ml each of 0.3 M NaCl and 0.6 M NaCl in 0.05 M phosphate buffer, pH 6.8, was applied and the flow rate was adjusted to 1 ml/min with a Beckman Accu-flo pump. One-hundred, 2-ml fractions were collected.

0.1 N acetic acid, the precipitate was immediately centrifuged and the methylase capacity of the enzyme was determined by dissolving the precipitate in 0.05 M Tris-HCl, pH 8.2. At saturation level the increase in the methylase capacity of the precipitated enzymes of normal, ovariectomized, and hormone-treated animals was 1.6-, 2.7-, and 1.6-fold, respectively; relative to the total methylase capacity of the original crude extracts of the three different uteri. The total methylase capacity of the precipitated enzymes from the three sources was the same (Figure 2). The supernatant from the pH 5 precipitation (tested after adjusting to pH 8.2) had no measurable enzyme activity, and its addition to the redissolved pH 5.0 precipitate resulted in an inhibition of methylase activity of the latter. It is evident from these results that the changes observed in the methylase capacity of extracts are due to different amounts of inhibitor(s) present

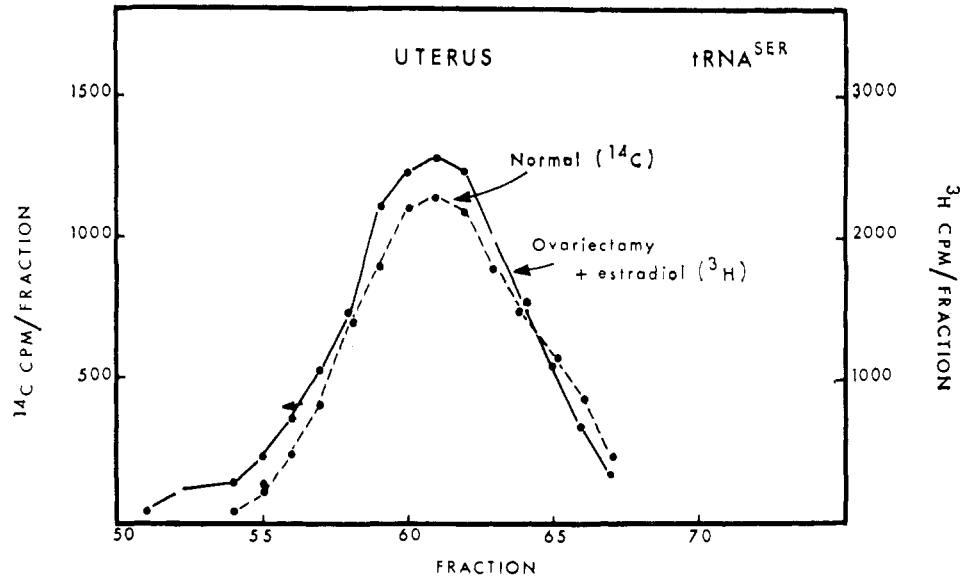

FIGURE 5: Elution profile on MAK of tRNA^{Ser} from normal, ovariectomized, and estradiol-treated pig uteri. Experimental details are described in Figure 4.

in these extracts. The enzymes from the liver of normal and ovariectomized animals had no detectable difference in the methylase capacity, nor was there any preferential increase in enzyme capacity in the fractions obtained by pH 5.0 precipitation (Figure 3). The data from these experiments provide an additional line of evidence for the constancy of the tRNA methylase capacities among individual animals.

Nature of the Inhibitor. The inhibitory activity (pH 5.0 supernatant) was destroyed by heating at 100° for 5 min and it was sensitive to trypsin. That the observed inhibition of tRNA methylases was not due to the action of ribonuclease on tRNA was determined by assaying for ribonuclease activity by preincubating tRNA with solutions of the inhibitor (pH 5.0 supernatant). The inhibitor was then inactivated by heat, and enzyme and *S*-adenosyl-L-methionine were added to the heated mixture. It is evident from Table I that preincubation of tRNA with the inhibitor did not result in a significant lowering of methylase activity on the substrate compared to a control, in which tRNA was preincubated with water instead of inhibitor. The inhibition cannot be ascribed to the cleavage of *S*-adenosyl-L-methionine either. The *S*-adenosyl-L-methioninase activity in the pH 5.0 supernatants was tested by the method of Gefter *et al.* (1966). There was less than 5% cleavage of substrate under the conditions of assay. No attempt was made to study whether the inhibitor is equally effective against the various base-specific methylases.

MAK Column Elution Profiles of Aminoacylated tRNA. Chromatographic comparisons of tRNA from normal and ovariectomized pig uteri, aminoacylated by glycine, alanine, valine, leucine, tyrosine, phenylalanine, methionine, arginine, and lysine, charged with rat liver enzyme did not show any difference in the elution profiles from MAK columns. The tRNA^{Ser} from ovariectomized animals eluted at a lower salt concentration than the tRNAs from the normal uterus (Figure 4). This was confirmed by reversing the labeled amino acids. However, the elution profile of tRNA^{Ser} from an ovariectomized animal following the administration of estradiol coincided with that of normal animals (Figure 5). No change in the population of tRNA^{Ser} was observed in liver following ovariectomy (Figure 6). It is interesting to note that methyl-deficient tRNA^{Phe} from *E. coli* elutes at a lower salt concentration from the MAK column than do the normal species (Revel and Littauer, 1965). On the other hand, Baliga *et al.* (1969) found that tRNAs from Novikoff hepatoma (which has elevated levels of tRNA methylases) eluted at higher salt concentration than those from normal rat liver. From the available data it is impossible to conclude whether the altered elution profile of tRNA^{Ser} from ovariectomized uteri stems from altered patterns of methylation.

Discussion

These studies demonstrate a specific effect of ovariectomy and of estradiol treatment on the tRNA methylases as well as on the population of tRNAs in the target organ. Increase in the amount of tRNA following the administration of estrogenic hormone has been observed in chick oviducts (Dingman *et al.*, 1969). Hacker (1969) has reported elevated levels of tRNA methylases in chick oviducts upon administration of massive doses of diethylstilbesterol. Unfortunately, however, no other organs were studied. Turkington (1969) studied the effect of hormones on the tRNA methylases in mammary epithelial cells and observed an elevation of methylase activity upon the administration of insulin and prolactin. However, here too, data on the nontarget organs are lacking.

The mechanism of hormonal influence on the enzymes, *i.e.*, whether by affecting the *de novo* synthesis of methylases or by the regulation of the activity of inhibitor(s) of the enzymes, is not known. It appears from the results presented earlier that the increase in the level of inhibitor following ovariectomy and its restoration to normal level upon administration of small doses of estradiol is closely associated

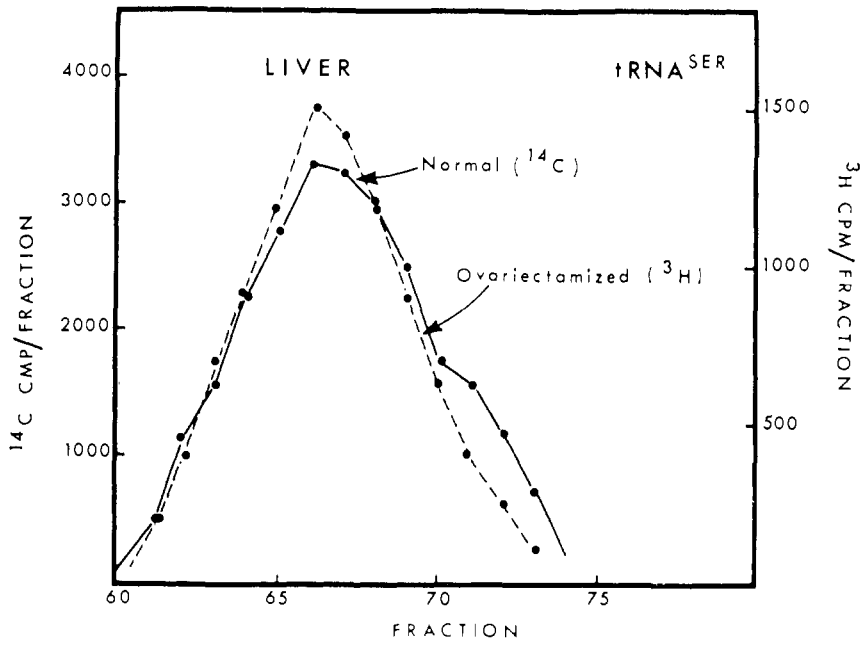

FIGURE 6: Liver $tRNA^{Ser}$ elution profile on MAK of normal and ovariectomized pig. Experimental details as described in Figure 4.

with the changes in the tRNA methylases. The inhibition of a specific enzyme could result in the production of a tRNA molecule with altered structural and possibly functional characteristics.

Alterations in tRNA populations have been observed in other systems under the influence of hormone; $tRNA^{Ser}$ in rooster liver during estrogen induced synthesis of phosphovitin (Mäenpää and Bernfield, 1969), $tRNA^{Leu}$ in liver, kidney, tail, and gills during induced metamorphosis in bullfrog tadpoles by triiodothyronine (Tonoue *et al.*, 1969) and $tRNA^{Tyr}$ in rat liver by thyroxine (Yang and Sanadi, 1969). However, the structural alterations of tRNA in these instances remain obscure. Methylation of tRNA affects its interaction with the charging enzyme (Shugart *et al.*, 1968), its coding response (Peterkofsky *et al.*, 1966), and its ability to attach to ribosomes (Gefter and Russell, 1969). Recently evidence for the translational control by tRNA in the synthesis of hemoglobin has been provided by Anderson and Gilbert (1969). Alterations in tRNA methylases under the influence of estrogenic hormone, may serve as a regulatory function for the protein-synthesizing systems in pig uteri.

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Levels of 5,6-Dihydrouridine in Relaxed and Chloramphenicol Transfer Ribonucleic Acid*

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ABSTRACT: Levels of the minor nucleosides dihydrouridine, pseudouridine, and ribothymidine in relaxed and chloramphenicol transfer ribonucleic acid of *Escherichia coli* and *Salmonella typhimurium* were determined. The levels were determined by labeling ribonucleic acid for several minutes at various times after uncoupling of ribonucleic acid and protein synthesis and determining radioactivity in nucleosides after bidimensional thin-layer chromatography. In agreement with the literature, pseudouridine and ribothymidine are present at normal levels in relaxed and chloramphenicol transfer ribonucleic acid. However, progressively less label enters dihydrouridine. Preparations of transfer ribonucleic acid, labeled for about 15 min after 2 hr of ribonucleic acid synthesis without protein synthesis, have only one-half of the normal level of labeled dihydrouridine. The data probably indicate that transfer ribonucleic acid deficient in dihydrouridine is synthesized. Essentially none of the uridine moieties of transfer ribonucleic acid destined to become dihydrouridine, pseudouridine, or ribothymidine are derived from cytidine moieties.

In bacteria, RNA synthesis will occur in the absence of protein synthesis under special experimental conditions. Withholding a required amino acid from an *Escherichia coli* strain carrying the relaxed allele of the RNA control gene leads to net RNA synthesis without protein synthesis (Borek *et al.*, 1955; Stent and Brenner, 1961). Also, a high concentration of chloramphenicol (200 μg/ml) effectively stops protein synthesis while permitting RNA synthesis with or without required amino acids. In *E. coli*, rRNA[1] synthesized under either of these conditions is undermethylated (Dubin and Gunalp, 1967; Gordon and Boman, 1964; Sypherd, 1968) and rRNA synthesized in the presence of chloramphenicol was reported to be deficient in pseudouridine (Dubin and Gunalp, 1967). The rRNA so synthesized is considered to be precursor rRNA.

The methylated base and pseudouridine levels of tRNA synthesized during treatment of *E. coli* with chloramphenicol are essentially normal, according to Dubin and Gunalp (1967). Data of Mandel and Borek (1963) indicate that RNA synthesized in an RC^{rel} strain of *E. coli* under relaxation conditions has normal levels of methylated constituents and pseudouridine. Their studies were done on total RNA but probably reflect the levels of minor nucleosides in tRNA since these levels are higher in tRNA than rRNA.

Thus, it appears that *E. coli* tRNA, in contrast to rRNA, is unaffected by uncoupling of RNA and protein synthesis, whether this is accomplished by use of an RC^{rel} strain or treatment with chloramphenicol. However, data reported by Mandel and Borek (1963) for pseudouridine levels and by Dubin and Gunalp (1967) for pseudouridine and methylated nucleoside levels were obtained by labeling RNA for short or long periods immediately after chloramphenicol treatment or imposing relaxation conditions. Since that procedure tends to mask effects on minor nucleoside levels developing only after conditions are well established, we reinvestigated relaxed and chloramphenicol tRNA by labeling for relatively short periods at various times after initiation of the particular condition of RNA synthesis.

This report concerns only the minor nucleosides dihydrouridine, pseudouridine, and ribothymidine. The biochemistry of pseudouridine (Ginsberg and Davis, 1968; Goldwasser and Heinrikson, 1966; Kusama *et al.*, 1966) and of the methylation of RNA (Borek and Srinivasan, 1966) has been under study for several years. However, dihydrouridine was first reported as a naturally occurring constituent of RNA ($tRNA^{Ala}$ from yeast) by Madison and Holley (1965).

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[1] Abbreviations used are: rRNA, high molecular weight rRNA; 5S RNA, low molecular weight ribosomal RNA; RC^{rel}, relaxed allele of the RNA control gene; RC^{str}, stringent allele of the RNA control gene; Hrd, dihydrouridine.