Transfer Ribonucleic Acid Methyltransferases During Hormone-Induced Synthesis of Phosvitin*

Laura Livingston Mays and Ernest Borek

ABSTRACT: During estrogen-induced phosvitin synthesis by rooster liver, the tRNA methyltransferase capacity of the liver is decreased. Analysis of hydrolyzed tRNA methylated *in vitro* showed that base-specific enzymes are not uniformly affected. The enzyme activities producing N^2-methylguanine are higher in overall capacity after estrogen treatment, while those producing a number of other methylated bases are lower. The extracts were fractionated on hydroxylapatite columns. Two of the three peaks of methyltransferase activity were about one-fourth as active in extracts of estrogen-treated roosters as in normal roosters. The tRNA methylated *in vitro* by each peak was analyzed. While N^2-methylguanine methyltransferase was present in all three peaks, only one of these activities was increased after hormone treatment. All enzymes producing other methylated bases were either constant or diminished after hormone treatment.

The synthesis of phosvitin, an egg yolk protein, can be induced in the liver of the rooster by massive doses of estrogens. This is a highly useful biological system for studying the *de novo* synthesis of a protein, because phosvitin can be readily characterized by its extremely high serine and phosphate content.

Mäenpää and Bernfield (1971) have observed an increased level of two of the four isoaccepting $tRNA^{Ser}$ species in the liver of roosters after the administration of estrogen. The appearance of a novel isoaccepting $tRNA^{Ser}$ had been observed to be a concomitance of estrogen *deprivation* in our laboratory in another biological system (Sharma and Borek, 1970). This $tRNA^{Ser}$ appears in the uterus of ovariectomized pigs and disappears from that organ after the administration of physiological doses of estradiol. We have also observed another phenomenon in the uterus as a sequela of ovariectomy: the castration is followed by a sharp reduction in the total capacity of the tRNA methyltransferases, enzymes which modify the structure of tRNA. The administration of estrogen restores the methylating capacity of extracts of the uterus to normal.

The tRNA methyltransferases have been found to undergo extensive qualitative and quantitative alterations in several biological systems undergoing shifts in regulatory processes. (For a list of these see, Borek, 1971.)

Examination of the tRNA methyltransferases in the liver of the rooster after the administration of estrogens revealed an unexpected pattern, contrary to the effect of physiological doses of estradiol in the mammalian target organ, the uterus, where the administration of the hormone results in a doubling of total enzyme capacity (Sharma *et al.*, 1971). Administration of massive doses of estrogen to the rooster reduced the total methyltransferase capacity of the liver. However, quantitation of the base-specific enzymes revealed that they are not uniformly affected; some increase in capacity, some decrease.

Materials and Methods

Animals. White Leghorn roosters of 2–3 lb weight were used throughout these experiments, with the exception of the saturation studies, when both Rhode Island Red and White Leghorn roosters were used and found to show no difference.

Hormones. Estradiol benzoate (Calbiochem) or diethylstilbestrol (Mann) were used in these experiments. Estradiol was administered at 10 mg/kg body weight, injected into the breast muscles as a suspension in sesame oil. Diethylstilbestrol was administered the same way at a dose of 25 mg/kg.

Purified tRNAs. Purified tRNAs for glutamic acid and arginine from *Escherichia coli* were provided for us by the Oak Ridge National Laboratories.

Rooster tRNA. Rooster liver tRNA was prepared by the method of Brunngraber (1962) with isopropyl alcohol fractionation according to Rogg *et al.* (1969).

Preparation of Extracts. Fresh rooster liver was homogenized in six volumes of cold 0.25 M sucrose, 0.02 M Tris-HCl (pH 8.2), 0.01 M $MgCl_2$, and 0.005 M β-mercaptoethanol by means of a motor-driven Potter-Elvejhem homogenizer with a Teflon pestle. The extract was then centrifuged, first at 32,000*g* for 10 min, and then at 105,000*g* for 1 hr. The high-speed supernatant served as the source of the enzymes. Extracts for use on hydroxylapatite columns were prepared identically except that only four volumes of buffer were used.

Assay for tRNA Methylase Activity. The basis of the assay is the incorporation of radioactive methyl groups from *S*-adenosylmethionine into *E. coli* B tRNA, a heterologous substrate. The reaction mixture contained the following in a total volume of 0.2 ml: 10 μmoles of Tris-HCl (pH 8.2), 1 μmole of β-mercaptoethanol, 1 μmole of $MgCl_2$, 0.1 μCi of *S*-adenosylmethionine (methyl-^{14}C label, specific activity 55 Ci/mole), 40 μg of *E. coli* B tRNA, and extract containing less than 2 mg of protein. Control reactions contained no tRNA. After incubation at 37° for 1 hr, reactions were stopped by chilling in an ice–water bath, and 0.1 ml of 20% potassium acetate (pH 5) and 1.0 ml of cold 95% ethanol were added. The reaction tubes were shaken and placed at −20° for at least 10 min before collection. The precipitates were collected on GF/A Whatman glass fiber filters using a Millipore sampling manifold. The filters were dipped in 0.10 M Na_2EDTA before use to reduce nonspecific adsorption. The collected samples were washed three times with ice-cold 67% ethanol containing 0.1 M sodium acetate. They were then glued to planchets, dried under an infrared lamp, and counted in a Nuclear-Chicago gas-flow counter at an efficiency of 25%.

* From the University of Colorado Medical Center Department of Microbiology, Denver, Colorado 80220. *Received July 1, 1971.* Our work was supported by a contract from the Atomic Energy Commission and by a grant from the American Cancer Society.

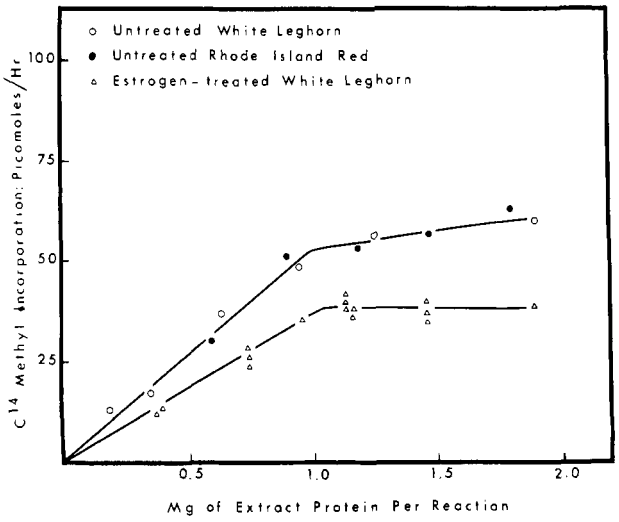

FIGURE 1: Methyltransferase saturation curves for extracts of normal and estrogen-treated roosters.

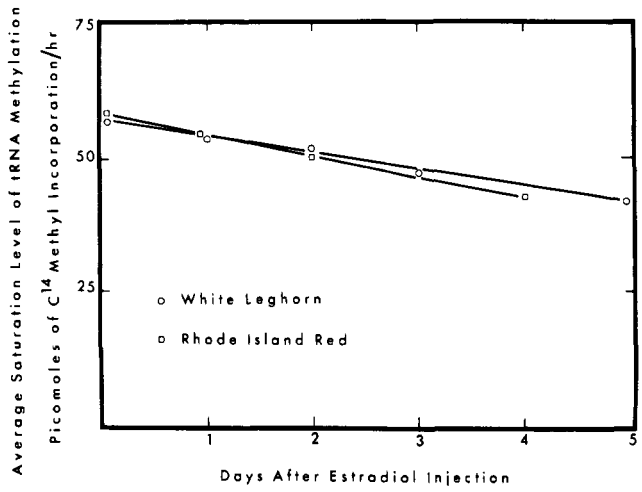

FIGURE 2: Rooster liver tRNA methyltransferase saturation levels following estrogen injection.

Protein and RNA Assays. Protein was assayed by the method of Lowry *et al.* (1951) and RNA by the orcinol method of Dische and Schwartz (1937).

Base Analysis. Enzyme at a predetermined saturating level was added to reaction mixtures as above, with all components increased tenfold. After 4-hr incubation at 37°, the reactions were stopped by chilling, and 2 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline was added. The tubes were well mixed and allowed to stand at 4° overnight. The tubes were then centrifuged and the water layer was removed. The RNA was precipitated from the water layer by the addition of 0.1 volume of 20% potassium acetate at pH 5.0 plus 2 volumes of absolute ethanol. The tubes were mixed and placed at −20° overnight. The precipitate was collected, and washed sequentially with 67% ethanol containing 1 M sodium acetate, 95% ethanol, absolute ethanol, and ether. The precipitate was dried, and an amount equivalent to 0.2 mg of tRNA was hydrolyzed in 1 N HCl for 1 hr at 105° in a sealed ampoule. The hydrolysate was then analyzed by two-dimensional thin-layer chromatography as described by Björk and Svensson (1967).

Hydroxylapatite Columns. Bio-Gel HT (Bio-Rad) was transferred to 2.0 mM potassium phosphate buffer at pH 7.5. A 20 × 1.5 cm column was poured and washed extensively with this buffer. One day before its use, the column was equilibrated with cold buffer A (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol, and 0.2 mM potassium phosphate, pH 7.5.) An aliquot of 25 ml of the 105,000*g* supernatant from 10 g of liver, prepared as described, was applied to the column. After the sample was adsorbed, 100 ml of buffer A was passed through the column at a flow rate of about 7 ml/hr and collected in 10-ml fractions. Then a linear gradient was applied using 200 ml of buffer A and 200 ml of buffer B (buffer A with 500 mM potassium phosphate, pH 7.5). Elution was performed as a flow rate of about 7 ml/hr and fraction volumes of 3 ml were collected.

Results and Discussion

tRNA Methyltransferase Capacity. The methylation of tRNA by crude extracts from various sources reaches a saturation level with increasing extract in a manner characteristic of the source of the enzyme. Data from several such experiments using extracts of livers from intact roosters and livers from roosters 3 days after estradiol injection are plotted in Figure 1. The level of protein required for saturation was approximately 1 mg/40 μg of *E. coli* B tRNA for both types of extract, but the saturation level of methyl groups incorporated decreased from 58 to 43 pmoles per hr after estrogen treatment.

Data from at least three such experiments were used to obtain the average saturation levels of tRNA methylation during a 5-day period after estradiol injection, as plotted in Figure 2. There is no apparent difference between the responses of White Leghorn and Rhode Island Red roosters to this hormone; both show a linear decrease in the saturation level of the methyltransferases during this period.

Diethylstilbestrol injection into White Leghorn roosters produced a similar decrease in methylation during the same time period: 4 days after diethylstilbestrol injection the saturation level was 43 pmoles/hr as compared to 58 pmoles/hr for the untreated roosters.

Base-Specific Enzymes. The total methylated tRNA was examined to assess whether the methylation capacity of the various base-specific enzymes had decreased uniformly. Table I shows analytical results from the *in vitro* methylation of *E. coli* B tRNA by liver extracts of roosters before and after estrogen treatment. The percentage of the total label in N^2-methylguanine increased after estrogen treatment, whereas the percentage in the other bases decreased. Clearly, enzymes producing the various methylated bases were not equally affected by the estrogen treatment.

When the values from Table I were converted to specific activities for the individual bases, the data plotted in Figure 3 were obtained. The potential to form N^2-methylguanine, in the substrate *E. coli* B tRNA, increased during 5 days after estrogen treatment, while the overall capacity to produce other methylated bases declined.

Studies with Purified tRNA Preparations. Recent experiments in Nishimura's laboratory have demonstrated that the tRNA methyltransferases have, not only base specificity, but site and tRNA species specificity as well. A particular guanine tRNA methyltransferase will methylate only a guanine in a specific site and only in some tRNAs. Apparently the tertiary

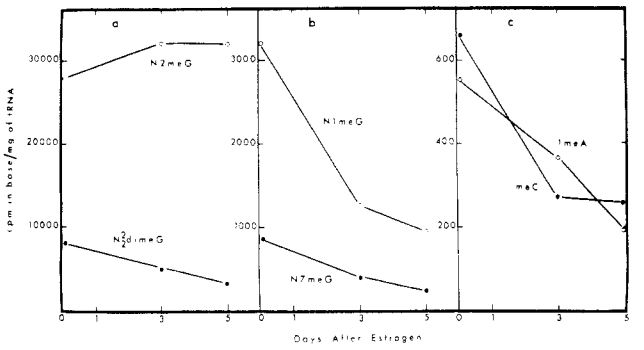

FIGURE 3: Distribution of [^{14}C]methyl groups per milligram of tRNA into different methylated bases after estrogen treatment of roosters.

structure of the tRNA is the determinant of receptivity (Kuchino and Nishimura, 1970). Nishimura's studies imply that a putative base-specific tRNA methyltransferase may really be a family of enzymes, the extent of whose multiplicity is undetermined at the present time. It seemed of interest, therefore, to study whether the effect of estrogen is the same on all the interactions of the enzymes. This was done by exposing purified preparations of $tRNA^{Arg}_{E.\,coli}$ and $tRNA^{Glu}_{E.\,coli}$ to the enzyme extracts from rooster liver before and after the administration of estrogen.

In Table II the relevant data from these experiments are presented. It is evident that the estrogen-induced elevation in the capacity to produce N^2MeG is not a uniform effect on all species of tRNA. The study of the mechanism of this extreme specificity of hormonal action should be challenging.

TABLE I: Base Compositions of *in Vitro* Labeled tRNA Produced by Methylating Enzymes of Untreated and Estrogen-Treated Roosters.[a]

Methylated Base	% of Label in Base,[b] ± Standard Deviation; No. of Determinations[c] in Parentheses		
	Untreated (3)	3 Days after (6)	5 Days after (2)
N^2-Methylguanine	66.9 ± 2.5	80.6 ± 3.2	84.6 ± 0.9
$N_2{}^2$-Dimethylguanine	18.7 ± 2.5	12.0 ± 1.8	9.8 ± 4.2
N^1-Methylguanine	7.3 ± 1.1	3.7 ± 1.1	2.6 ± 1.3
N^6-Methylguanine	2.0 ± 0.2	1.0 ± 0.2	0.7 ± 0.1
1-Methyladenine	1.3 ± 0.4	0.9 ± 0.8	0.5 ± 0.4
Methylcytidylate	1.6 ± 1.5	0.7 ± 0.3	0.7 ± 0.1

[a] Samples of tRNA were methylated by extracts of different roosters. The RNAs were then hydrolyzed and analyzed by thin-layer chromatography as described in the Materials and Methods Section. [b] Total counts loaded on plates varied from 3700 to 10,200. The average proportion of these counts recovered in the bases was 82% for untreated roosters, 80% for roosters 3 days after estrogen, and 84% for roosters 5 days after estrogen. The counts in 6-methyladenine and in thymine were very low and variable between identical preparations. [c] Each determination was made using tRNA methylated *in vitro* by extract from a separate rooster.

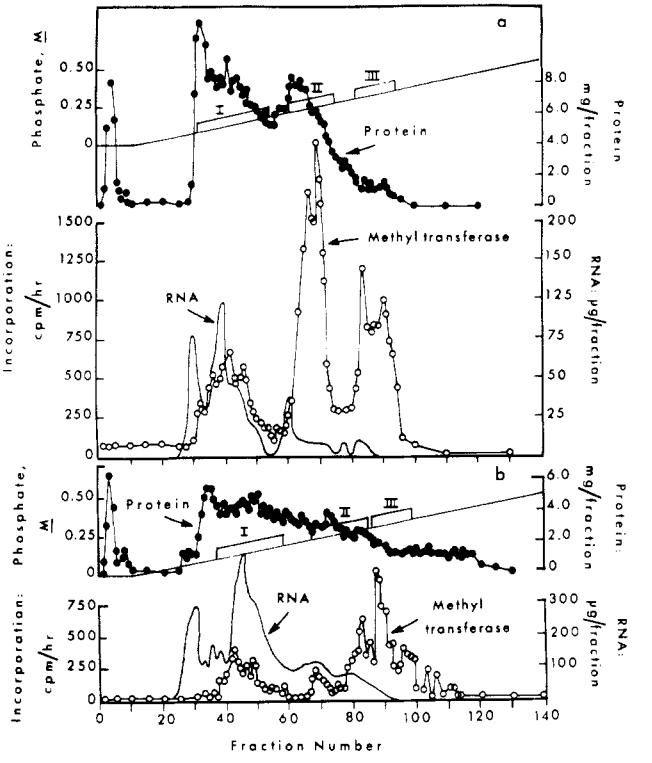

FIGURE 4: Elution of protein and methyltransferase activities from hydroxylapatite columns loaded with extracts of liver from (a) normal and (b) estrogen-treated roosters.

Fractionation of Methyltransferases on Hydroxylapatite. As an approach to this study the enzymes were fractionated on hydroxylapatite columns. The elution of protein, RNA, and methyltransferase activity from a column loaded with extract from a normal rooster can be seen in Figure 4a. Three main regions of activity, labeled I, II, and III, were found. The activity labeled I coincided with a peak of RNA, and with a large amount of protein. The activity labeled II eluted immediately following a large protein peak which absorbs at 408 mμ and may be hemoglobin. There was little RNA in fraction II. The activity labeled III was obtained following the elution of most of the protein and the RNA.

To characterize the enzyme activities individual fractions

TABLE II: The Production of N^2MeG in Purified Preparations of tRNA by Extracts of Treated and Untreated Roosters.[a]

	Normal Rooster Liver		Rooster Liver, 3-Days Postestrogen	
N^2MeG in $tRNA^{Arg}_{E.\,coli}$	% of Label in N^2MeG[b] 92.5, 90.3	Av 91.4	% of Label N^2GeG[b] 86.4, 88.77	Av 87.5
N^2MeG in $tRNA^{Glu}_{E.\,coli}$	64.1, 73.5	68.8	42.8, 53.2	48.0

[a] RNAs were hydrolyzed and examined by thin-layer chromatography as described in the Materials and Methods Section. [b] Each value given is for RNA methylated by an extract of a separate rooster. Average per cent recovery for normal roosters was 80%; that for treated roosters was 85%.

TABLE III: Effect of Estrogen Treatment of Roosters on the Formation of N^2-Methylguanine in Unfractionated or Arginine-Specific tRNA by Methyltransferases of the Three Hydroxylapatite Peaks.[a]

Hydroxylapatite Methyltransferase	cpm in N^2-Methylguanine/mg of tRNA per hr	
	Unfractionated tRNA	Arg tRNA
Peak I		
Untreated	4,050	6,604
3 days after estrogen	3,810	300
Peak II		
Untreated	159,432	192,736
3 days after estrogen	95,172	67,599
Peak III		
Untreated	43,896	50,970
3 days after estrogen	109,572	72,842
Sum of 3 peaks		
Untreated	207,376	250,410
3 days after estrogen	208,554	140,741

[a] Peaks were pooled as marked in Figure 4a,b on the basis of the phosphate concentration at which they eluted.

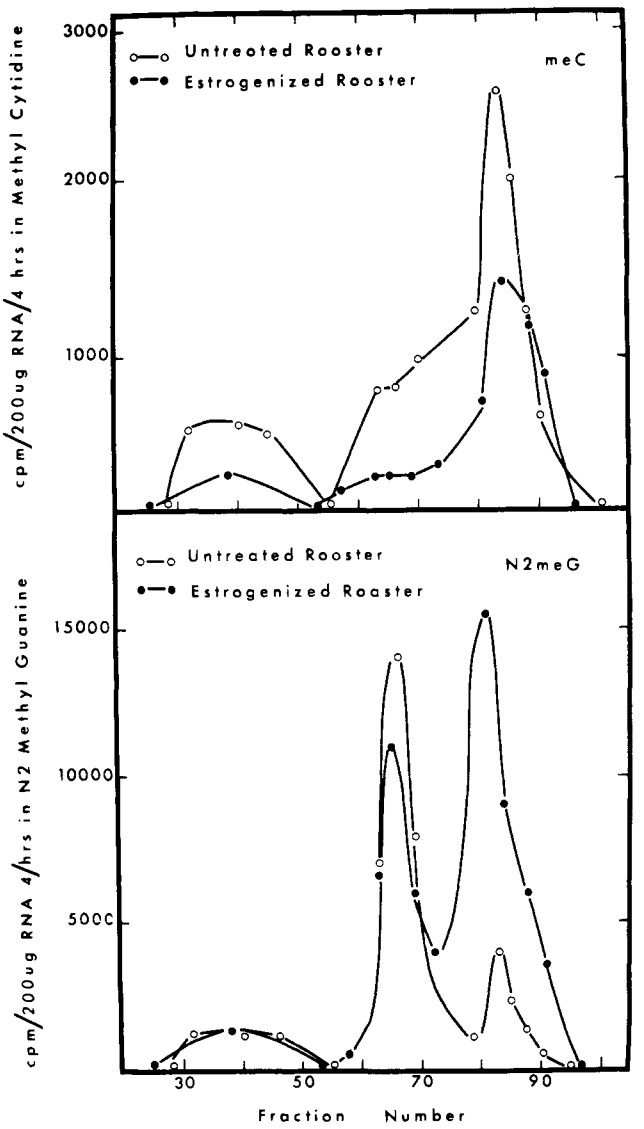

FIGURE 5: Production of N^2-methylguanine and methylcytidine by hydroxylapatite peaks of extracts of treated and untreated roosters.

were allowed to methylate large amounts of *E. coli* B tRNA. The tRNA was then analyzed for its methylated bases as described in the Methods section. Activity I includes enzymes producing all of the bases, but the enzymes are not uniformly distributed within the region; N_2^2-dimethylguanine methyltransferase precedes 1-methyladenine methyltransferase, for example. It should be noted that a very small amount of thymine production is catalyzed by this region and by the region of activity III, but the substrate will not accept many methyl groups on uracil, and so the results are not presented since they are extremely low and variable. Enzymes of region I occasionally produced 6-methyladenine in low levels as well. Activity II includes all of the activities producing derivatives of guanine plus 1-methyladenine methyltransferase. In contrast to activity I, however, activity II primarily produced one methylated base, N^2-methylguanine. The product of activity III was almost exclusively made up of N^2-methylguanine and methylcytidine in approximately equal amounts. These two methyltransferases were occasionally slightly resolved by hydroxylapatite columns.

The result of an identical fractionation of extract from a rooster liver 3 days after the rooster had been injected with estradiol can be seen in Figure 4b. The methyltransferase activity of region I again coincided with a peak of RNA, whose amount was increased about fourfold over that in the untreated rooster liver extracts. The activities in region I and region II were diminished to about one-fourth of their former value, while the activity of region III was diminished only slightly by estradiol treatment. The diminution in region I was particularly marked with respect to methylcytidine and 11-methyladenine. The diminution in region II was due to decreased production of N^2-methylguanine, its major product. The N^2-methylguanine methyltransferase of region III increased greatly (Figure 5b), while the methylcytidine methyltransferase decreased to about one-half its former value (Figure 5a).

The difference between the unfractionated and arginine-specific tRNAs as substrates was clarified by the data in Table III. With each substrate, estrogen caused a decrease in N^2-methylguanine production by peaks I and II and an increase by peak III. When the three activities were summed, however, the unfractionated tRNA showed an apparent increase in the production of this base, while the $tRNA^{Arg}_{E.\,coli}$ showed an apparent decrease after estrogen treatment.

Isolated Rooster tRNA as a Substrate. The tRNA was isolated from livers of untreated roosters and roosters 3 days after estradiol injection. Each of the tRNA preparations was tested as a substrate for enzymes from treated and from untreated roosters. In common with known homologous methyltransferase and tRNA interactions (Kerr, 1970; Borek, 1969), the tRNAs were poor substrates for the homologous enzymes. Nevertheless, it was possible to detect somewhat more sites for methylation in the tRNA from estrogen-treated roosters (Table IV). The increased methyl acceptance after estrogen could reflect a difference in the amount of nascent, undermethylated tRNA in the two kinds of roosters, or as has been observed before, prior methylation *in vivo* can increase the

TABLE IV: Rooster tRNA as a Substrate for Rooster Methyltransferases.[a]

Source of Enzymes	[^{14}C]Methyl Group Incorporation in pmoles/400 μg of RNA per 4 hr		% of Label Incorporated into N^2-Methylguanine	
	tRNA from Untreated Rooster	tRNA from Estrogen-Treated Rooster	tRNA from Untreated Rooster (%)	tRNA from Estrogen-Treated Rooster (%)
Untreated rooster	15	27	58.0	83.2
Estrogen-treated rooster	14	18	73.7	80.4

[a] The isolation of tRNA is described in the text.

receptivity of a tRNA in *in vitro* reactions, presumably because of alterations in tertiary structure produced by the previous methylation (Srinivasan and Borek, 1964).

In addition, the tRNA from estrogen-treated roosters yielded a greater amount of N^2-methylguanine with either set of enzymes *in vitro* (Table IV). The increment in total N^2-methylguanine methyltransferase activity after estrogen treatment (Figure 3a) could be expected to saturate the methyl-accepting sites of this substrate *in vivo*, and consequently it might be expected to be a poorer substrate for the formation of N^2-methylguanine *in vitro*. However, if some nascent tRNA were present after estrogen treatment, the results which were obtained might be expected.

Whether these complex modulations by hormones of the *in vitro* activity of the tRNA methyltransferases exert any influence on protein synthesis is obscure at present. The mechanism of such influence, if it exists, is even more obscure. The large variety of changes in the tRNA methyltransferases in so many different biological systems posed a restrictive question: are there enough possible permutations in the structure of tRNA to serve a regulatory role in the synthesis of so many different specific proteins? However, a very important recent finding by Jacobson (1971) releases the functional capacity of tRNA from the confines of its role in protein synthesis. Jacobson has demonstrated that an isoaccepting $tRNA^{Tyr}$ in a mutant of *Drosophila* inhibits the enzyme tryptophan pyrrolase. Thus a brand new role of tRNA, totally unrelated to protein synthesis has emerged. The potential variation of the structure of tRNAs for such roles is enormous.

Acknowledgments

We thank Dr. Sylvia J. Kerr and Dr. Opendra K. Sharma for their help during the course of this work.

References

Björk, G. R., and Svensson, I. (1967), *Biochim. Biophys. Acta 138*, 430.

Borek, E. (1969), Exploitable Molecular Mechanisms and Neoplasia, Baltimore, Md., Williams & Wilkins, p 163.

Borek, E. (1971), *Cancer Res. 31*, 596.

Brunngraber, E. F. (1962), *Biochem. Biophys. Res. Commun. 8*, 1.

Dische, Z., and Schwartz, K. (1937), *Mikrochim. Acta 2*, 13.

Jacobson, K. B. (1971), *Nature New Biol. 231*, 17.

Kerr, S. J. (1970), *Biochemistry 9*, 690.

Kuchino, Y., and Nishimura, S. (1970), *Biochem. Biophys. Res. Commun. 40*, 306.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Mäenpää, P. H., and Bernfield, M. R. (1971), *Biochemistry 10*, 4927.

Rogg, H., Wehrli, W., and Staehelin, M. (1969), *Biochim. Biophys. Acta 195*, 13.

Sharma, O. K., and Borek, E. (1970), *Biochemistry 9*, 2507.

Sharma, O. K., Kerr, S. J., and Lipshitz-Wiesner, R., and Borek, E. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 167.

Srinivasan, P. R., and Borek, E. (1964), *Biochemistry 3*, 616.