Another explanation of the data is that an enzyme is synthesized and it then produces a vital component of the receptor, perhaps a ligand which can activate an inactive receptor. No evidence exists for a ligand being bound to the activated receptor; however, our knowledge of its chemistry either with or without bound estrogen is too meager to eliminate this possibility.

The accumulating evidence that the receptor consists of subunits (Erdos, 1968; Jensen *et al.*, 1969) is compatible with the idea of a conformational change being induced to yield an active receptor from an inactive form.

A reciprocal relationship between replenishment and the loss of estrogen from the nucleus is intriguing but far from clear. Such a relationship would involve a lag of approximately 2 hr and is supported principally by the observation that, during *in vitro* incubations following estrogen administration, neither loss of estrogen from the nucleus nor replenishment occurs.

The relatively long half-life of the estrogen receptor in the absence of estrogen indicates a slow rate of degradation. Thus, a reasonable change in the rate of degradation would still not not be expected to bring the rapid changes in receptor concentrations seen in this study. The degradation rate of the receptor could, however, be markedly changed when the receptor is combined with estrogen or moves into the nucleus.

If the estrogen binding protein of the uterus is truly a receptor molecule with its accompanying physiological importance, then the regulation of the receptor itself is bound to be of interest. The studies reported here suggest that the regulation of the receptor will be as complicated to decipher as the mechanism of action of the receptor.

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Transfer Ribonucleic Acid Methylase Activity in the Developing Pig Brain*

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ABSTRACT: tRNA methylase activity in whole brain preparations from adult pigs was found to be very low (~10 $\mu\mu$moles/30 min per mg of protein), whereas examination of five separate regions, frontal cortex, cerebellum, pons medulla, midbrain, and corpus callosum, revealed some regions to be three to four times more active than that reported for the whole brain preparation.

Methylase activity was found to be high in fetal brain and to decrease in older animals after birth. A tRNA methylase inhibitor was also found in fed newborn pigs as early as 12 hr after birth and in adult animals. It was absent from fetal tissue and its synthesis or activation could be delayed for up to 48 hr in newborn pigs fasted immediately after birth.

Transfer RNAs have molecular weights between 25,000 and 30,000 and contain in addition to the normal nucleotides over 30 minor bases. Some of these are derived by the methylation of the 4 main bases at the polynucleotide level by the tRNA methylase enzymes. These enzymes are base specific, site specific, and evidence is accumulating that indicates that they are sequence specific as well (Srinivasan and Borek, 1963; Baguley and Staehelin, 1969; Kuchino and Nishimura, 1970).

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The activity of tRNA methylase enzymes has been shown to increase and decrease in many cell populations. Increased methylase activity has been reported in certain human tumors (Tsutsui *et al.*, 1966), in liver of chicks infected with the oncogenic Marek's disease virus (Mandel *et al.*, 1969), and in SV-40 and adenovirus-12 hamster tumors (McFarlane and Shaw, 1968; Mittleman *et al.*, 1967). Recently Tidwell (1970) has reported tRNA synthesis and tRNA methylation to be a weakly coupled event in regenerating rat liver and proposes that methylation of tRNA may be regulated at the enzyme level. The importance of proper methylation of tRNA and its possible control of protein synthesis is presently being investigated (Tidwell, 1970). Since control of the extent of tRNA methylation appears to be at the enzyme level, the role of inhibitors as regulators of methylase enzymes has been and is continuing to be explored (Wainfan *et al.*, 1965; Kerr, 1970; Chaney *et al.*, 1970).

Due to the importance of tRNA methylation as a possible regulator of central nervous system protein synthesis, we report the activities of methylase enzymes in the developing pig brain. In addition, a tRNA methylase inhibitor(s) was found in fed newborn and adult animals. It was absent from fetal tissue and totally fasted newborn animals.

Experimental Section.

Materials. Yorkshire, Duroc, and Hampshire pigs were farrowed at the University of Illinois animal research facilities. Preterm animals were delivered by cesarean section from pregnant pigs of known farrowing dates based on a 114-day period of gestation. At birth, newborns were fed on the sow or kept totally fasted until the start of the experiment. Fasted animals were kept warm with heating lamps in an isolated area with free access to water.

[^{14}C]Methyl-*S*-adenosylmethionine (specific activity 35–55 mCi/mmole) was purchased from International Chemical and Nuclear Co., Irvine, Calif.

Escherichia coli B tRNA was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

Protein was determined by the method of Mokrash and McGilvery (1956).

Assay of tRNA Methylase. The standard assay mixture contained 19 μmoles of Tris-HCl buffer (pH 8.5), 5 μmoles of $Mg(C_2H_3O_2)_2$, 16 μmoles of mercaptoethanol, 16 mμmoles of [^{14}C]methyl-*S*-adenosylmethionine (containing *ca.* 0.96×10^6 cpm), 300 μg of *E. coli* B tRNA, and varying amounts of enzyme in a total volume of 0.6 ml. The reaction was started by the addition of enzyme supernatant and incubated for 30 min at 37°. Control reactions, without tRNA, were treated identically to determine background methylation levels. The reaction was stopped after 30 min by the addition of ice-cold 6% trichloroacetic acid. The precipitate was collected by filtration on a Millipore filter and washed twice with cold 6% trichloroacetic acid to remove unincorporated [^{14}C]methyl-*S*-adenosylmethionine. The filters were then transferred to vials and counted in a Packard liquid scintillation counter. An alternate method for determining the amount of [^{14}C]methyl incorporated into tRNA was also used and was based on the method of Manns and Novelli (1961). Reactions were stopped by pipetting 0.10 ml of assay mixture onto Whatman No. 3MM filter disks (2.5cm) and hot air-dried. Filters were washed twice in ice-cold 6% trichloroacetic acid, then twice in diethyl ether, and air-dried. Samples were then transferred to vials and counted.

Preparation of tRNA Methylase Supernatant. Brains were removed from sodium amytal (10 mg/100 g body wt) anesthetized animals and used fresh or stored at −60°. Pig brains were sectioned into the following areas: frontal cortex, cerebellum, midbrain, pons medulla, and corpus callosum. Brain sections were minced and homogenized in a Dounce homogenizer in five to nine volumes of ice-cold 0.25 M sucrose–0.01 M $MgCl_2$ buffer with six strokes of the loose pestle and four strokes of the tight pestle. The homogenate was centrifuged successively at 1000*g* for 10 min and 105,000*g* for 2 hr. A high-speed supernatant was prepared from the frontal cortexes of 5-day-old pigs and was brought to pH 5 by the addition of 1 N acetic acid and centrifuged at 10,000*g* for 10 min. The precipitate was washed once with 0.32 M sucrose–0.002 M EDTA (pH 5.0) and dissolved in 0.01 M Tris-HCl buffer (pH 8.2) containing 0.002 M EDTA and 0.005 M 2-mercaptoethanol. This solution was the source of inhibitor-free pH 5 enzyme. The pH 5 supernatant from the 10,000*g* centrifugation was divided into three portions. One portion was neutralized immediately, the second was dialyzed 18 hr against 0.25 M sucrose–0.1 M $MgCl_2$ buffer and then neutralized, and the third was kept at 2° for 18 hr and then neutralized. These served as the source of inhibitor solutions and were used in the stability experiments.

All assays were performed in duplicate and represent the mean of at least two animals per age group with the exception of assays of midbrain and corpus callosum which were done in duplicate on pooled samples.

Results

Comparison of tRNA Methylase Activity in Various Areas of Adult Brain. In Table I are the values obtained for methylase activities prepared from whole adult pig brain and from the frontal cortex, midbrain, pons medulla, cerebellum, and corpus callosum of adult pigs. It is not surprising that a low activity was found for the whole brain preparation when the relative mass of the frontal cortex and cerebellum is considered in comparison to the other three areas. It is interesting to note that fourfold differences in activity exist between the separate brain regions.

tRNA Methylase in Fetal, 5-Day-Old, 2-Week-Old, and Adult Brain. Table II compares the activities from the 105,000*g* supernatants of frontal cortex, cerebellum, pons medulla, midbrain, and corpus callosum of fetal (−10 days), immature (5 and 14 days) and from adult pigs. The decrease in activities observed between fetal and older tissue in the frontal cortex and cerebellum is very similar to that observed in whole rabbit brain (Kerr, 1970) and in whole rat brain preparations (Simon *et al.*, 1967). The presence of tRNA methylase inhibitors in pig brain may help to explain the differences observed in methylase activity at various stages of development as Kerr (1970) has demonstrated them to exist in adult rabbit brain.

Methylase Activity Assayed at Different Protein Concentrations. In Figure 1 are the results of tRNA methylase activity measured against increasing protein concentrations in the frontal cortex of fetal, 2-week-old, and adult pigs. The assay of the 105,000*g* preparations revealed that extracts from fetal brain had a much greater capacity to methylate than extracts from either the 2-week-old or adult tissue. Kerr (1970) demonstrated that at increased protein concentrations (1 mg/ml assay) adult rabbit brain did not methylate *E. coli* tRNA to the extent of the corresponding fetal tissue and that this was due to the presence of a tRNA methylase inhibitor. The decrease in the extent of methylation observed in the 2-week-old or adult pig extracts beyond a protein concentration of ap-

TABLE I: tRNA Methylase Activity in Adult Pig Brain.[a]

Specific Areas	Act.[b]
Whole brain	10
Frontal cortex	9
Cerebellum	12
Pons medulla	43
Midbrain	47
Corpus callosum	22

[a] High-speed extracts of adult pig brain were prepared as described in the text. [b] Activity is defined as μμmoles of [^{14}C]-methyl incorporated into tRNA per mg of protein per 30 min. Each reaction mixture contained 1.2–1.8 mg of protein.

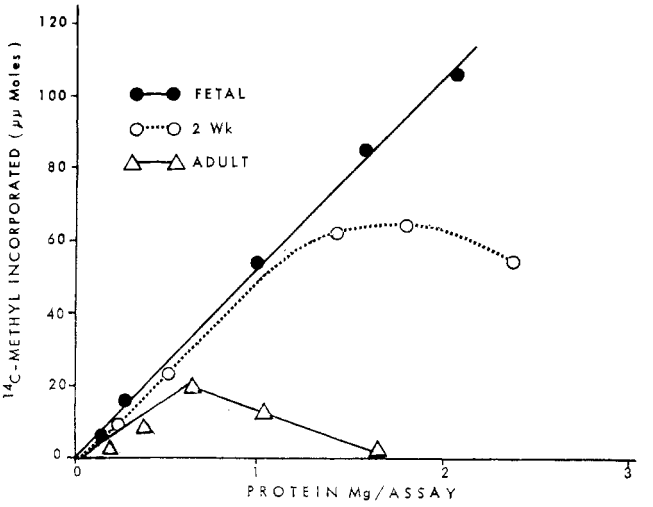

FIGURE 1: Methylation of tRNA by the 105,000*g* supernatants from fetal, 2-week-old, and adult pig frontal cortex. All tubes contained 300 μg of tRNA and various amounts of enzyme preparation. All tubes were incubated at 37° for 30 min.

proximately 1 mg/ml indicates the presence of an inhibitor whose action depends upon absolute concentration.

Inhibition of Fetal tRNA Methylase Activity by High-Speed Extracts from Adult Brain. Table III shows the inhibition of fetal methylase activity by 100,000*g* extracts prepared from five sections of adult pig brain. These data further demonstrate the presence of inhibitors in adult tissue and may help to explain the differences in methylase activity observed at various stages of development (Table II).

Separation of Inhibitor from tRNA Methylase Activity. The inhibitor from 5-day-old pig frontal cortex was separated from the methylase activity by the pH 5 precipitation described above. The data (Table IV) indicate that the inhibitor was separated by this procedure and remained in the pH 5 supernatant. It is interesting that standing at 2° or dialysis for 18 hr destroys the ability of the pH 5 supernatant to inhibit methylation. Although no attempt has been made to identify the specific methylase enzyme from the pH 5 precipitation, Kerr (1970) demonstrated that under identical conditions N^2-guanine monomethylase is isolated as the sole methylating enzyme in rabbit liver.

Instability of the Inhibitor in High-Speed Extracts of Frontal Cortex. The results presented in Table V indicate that the inhibitor is inactivated by standing at 2° for 18 hr and that the methylase enzyme is quite stable under these same conditions. Furthermore, these data also indicate that the action of the inhibitor depends on absolute concentration, since at low protein concentration, identical rates of methylation are observed.

Time Course of the Development of tRNA Methylase Inhibitor. Figure 2 presents the time course of inhibitor development when methylase activity was measured at increasing protein concentrations in the frontal cortex from fetal, fed newborns (12–60 hr), and adult pigs. It is apparent that the inhibitor was either synthesized or activated within the first 12 hr of life in the frontal cortex. The extent of inhibition is similar after 12–60 hr, but not the same, and the inhibition continues to increase to that level observed in the adult tissue.

TABLE II: tRNA Methylase Activity at Various Stages of Development in the Pig Brain.

	Activity[a]			
Sp Areas	Fetal	5-Day	2-Week-Old	Adult
Frontal cortex	54	56	44	9
Cerebellum	91	71	60	12
Pons medulla	48	41	26	43
Midbrain	29	46	40	47
Corpus callosum	29	42	33	22

[a] Activity is defined as μμmole of [^{14}C]methyl incorporated into tRNA per mg of protein per 30 min. Each reaction contained approximately 0.6 mg of protein except the adult which contained 1.2–1.8 mg of protein. Incubation volume was 0.5 ml.

TABLE III: Inhibition of Fetal tRNA Methylase Activity by Extracts from Various Sections of Adult Brain.

Additions[a]	[^{14}C]Methyl Incorp (μμmoles)	% Inhibn
Fetal frontal cortex	56.5	0
+Adult frontal cortex (0.34 mg)	29.0	48
+Adult cerebellum (0.34 mg)	27.0	52
+Adult midbrain (0.21 mg)	35.0	38
+Adult pons medulla (0.19 mg)	29.6	47
+Adult corpus callosum (0.17 mg)	32.2	43

[a] Each incubation assay contained 0.68 mg of protein of fetal high-speed extract and high-speed extract from the adult pig brain as indicated. Incubations were as described in the text.

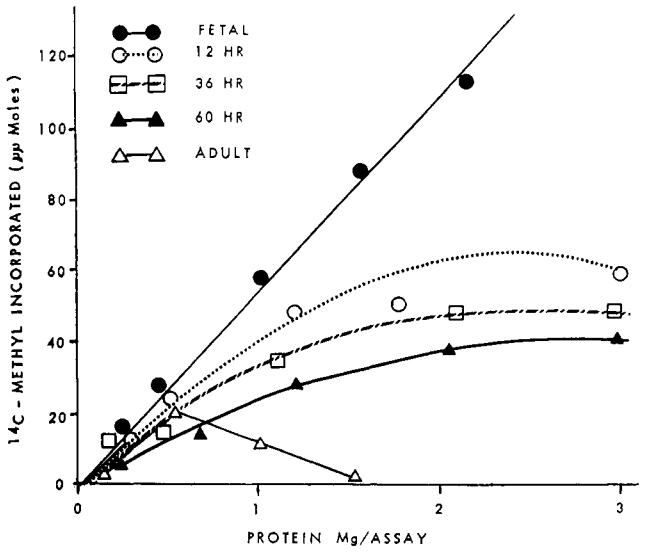

FIGURE 2: Methylation of tRNA by the 105,000*g* supernatants from the frontal cortex of fed newborn pigs. All tubes were incubated at 37° for 30 min.

tRNA Methylase in Fasted Newborn Pigs. Swiatek *et al.* (1970) have shown that withholding food from newborn pigs does not interfere or delay the development of hepatic gluconeogenic or glycolytic enzymes, but that it does change drastically the hormonal conditions of these animals (Swiatek *et al.*, 1968). Figure 3 shows the rate of tRNA methylation in the frontal cortex of both 24- and 48-hr-old totally fasted animals. The methylation is linear with protein concentration and indicates the absence of any inhibitor. Although both preparations appear to contain no inhibitor, the specific activity calculated for these fasted animals is less than that for the corresponding frontal cortex of fetal animals. It is possible that fasting may be delaying the appearance of only one of several inhibitors, but the linearity observed in both the fetal and fasted preparations tends to negate only one inhibitor to be affected. Kerr (1970) suggests several to be functional in rat and rabbit brains. It is also possible that the fasting state or fetal state may induce the activity of a substance that destroys the natural inhibitor.

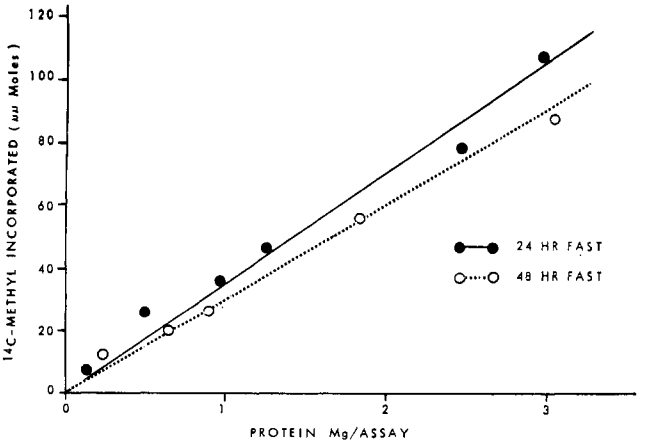

FIGURE 3: Methylation of tRNA by the 105,000*g* supernatants from the frontal cortex of totally fasted from birth newborn pigs. All tubes were incubated for 30 min at 37°.

TABLE IV: Separation of Inhibitor from tRNA Methylase Activity.[a]

Additions	[^{14}C]Methyl Incorp (μμmoles)
Frontal cortex pH 5 enzyme	8.3
+pH 5 supernatant (0.5 mg)	0
+pH 5 dialyzed supernatant (0.5 mg)	10.7
+pH 5 cold room control supernatant (0.5 mg)	10.6

[a] Incubation was in a total volume of 1.0 ml with 300 μg of tRNA for 30 min. Each assay contained 1.22 mg of frontal cortex pH 5 enzyme and the various pH 5 supernatants as indicated.

Discussion

The data presented support those studies which show higher tRNA methylase activity present in fetal brain tissue than adult brain tissue (Simon *et al.*, 1967; Kerr, 1970). Furthermore, the data support the conclusion that this is due to the presence of a methylase inhibitor(s) in adult tissue that is absent in fetal tissue (Kerr, 1970). Whether the differences in methylase activity between regions of the brain at any one stage of development are due to this same inhibitor remains to be completely resolved. It seems unlikely, however, that its sole presence can fully explain these differences since fetal tissue contains no inhibitor and large differences in methylase activity were observed between the various sections. In addition, the activities determined in the 5-day- and 2-week-old brain sections were determined at low protein concentrations (low inhibitor as well, Table II) and therefore in our estimation, these differences in methylase activity do represent different levels of enzyme and not necessarily varying amounts of inhibitor.

We have been able to demonstrate that the appearance of the inhibitor is a time-dependent process and that levels of

TABLE V: Effect of Freezing, Dialysis, and Standing on tRNA Methylase Activity Measured at Increasing Protein Concentrations.

Treatment[a]	[^{14}C]Methyl Incorp (μμmoles) Protein Concentration (mg) 0.2	0.4	0.8	1.6
Fresh supernatant	7	14	19	5
Frozen supernatant	7	13.1	20.4	33.8
Dialyzed supernatant	9.6	15.6	33.8	59.5
Standing at 2° supernatant	9.4	15.0	31.7	63.5

[a] A 100,000*g* supernatant was prepared from adult pig frontal cortex and assayed fresh and after 18 hr at −60°. A portion of the fresh supernatant was dialyzed for 18 hr against the homogenizing buffer at 2° and a portion kept at 2° in a cold room for 18 hr. Incubation conditions were as described in the text.

inhibitor are reasonably high as early as 12 hr after birth and increase dramatically as the animal becomes an adult. The data do not eliminate the birth process itself as being the triggering mechanism, however, studies carried out with fasted animals show that it is possible to delay the appearance of inhibitor activity by withholding food from the newborn animal for at least 48 hr.

Recently, Sharma and Borek (1970) have shown a tRNA methylase inhibitor to be under estradiol regulation in both rat and pig uteri. In pigs, growth hormone levels are high at birth and remain elevated in animals that are starved for 60 hr after birth. In contrast, growth hormone levels decrease in animals fed immediately after birth to those levels observed in adult pigs (Swiatek *et al.*, 1968). It would be interesting to determine whether the differences in levels of inhibitor in fed and fasted newborn pig brain are hormonally regulated or dependent on some other dietary factors as yet unexplained. Experiments are currently in progress to answer this question.

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Glutathione-Catalyzed Hydrogen Isotope Exchange at Position 5 of Uridine. A Model for Enzymic Carbon Alkylation Reactions of Pyrimidines*

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ABSTRACT: The effect of glutathione (GSH) on the exchange of hydrogen to deuterium at position 5 of uridine (Urd) was studied by using proton magnetic resonance spectroscopy. It was found that in D_2O solutions, at 80°, the rate of H-isotope exchange was enhanced in the presence of GSH and that the enhancement of the pseudo-first-order rate of exchange was proportional to the GSH concentration. The results obtained with GSH derivatives indicated the requirement of a free SH group for catalysis. The GSH-catalyzed H-isotope exchange showed a bell-shaped dependence on the OD^- ion concentration, suggesting that in the rate-determining step the ionized SH group of GSH reacts with the nonionized species of Urd. Ionization of Urd causes a substantial shielding of the proton at position 6, indicating the increased electron density of the 5,6-double bond, which may account for the lack of reactivity observed at high pD values. The results are consistent with a catalytic mechanism of H-isotope exchange involving the reversible addition elimination of the SH group of GSH across the 5,6-double bond of Urd.

The relevance of these findings to the mechanism of enzyme-catalyzed C-alkylation reactions of pyrimidine nucleotides is discussed.

Lomax and Greenberg (1967) and Yeh and Greenberg (1967) reported that thymidylate synthetase and deoxycytidylate hydroxymethylase, respectively, catalyze the exchange of hydrogen to tritium at position 5 of the substrates, deoxyuridylate and deoxycytidylate, and suggested that the labilization of H-5 may be the initial step in the overall substitution reaction. Both of these enzymic reactions belong to a group of metabolic transformations of pyrimidine nucleotides, which involve C–C-bond formation at position 5 of the pyrimidine ring. C-alkylation reactions of this type also include hydroxymethylation of deoxyuridylate (Roscoe and Tucker, 1966), the formation of the thymine, hydroxymethyluracil, and 5-carboxyuracil moieties of the nucleoside antibiotics, polyoxins (Isono *et al.*, 1969), and certain modifications of pyrimi-

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