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Effect of Estradiol-17β on Preprolactin Messenger Ribonucleic Acid Activity in the Rat Pituitary Gland†

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ABSTRACT: Rat pituitary RNA was translated in the wheat germ system. Preprolactin messenger RNA activity was estimated by adsorption of cell-free products to solid phase antiprolactin. When male rats were injected for 4 days with estradiol-17β, pituitary preprolactin mRNA activity was increased 2.5- to 3.0-fold over controls. This increase was evident when either total RNA, poly(adenylic acid) RNA, or polysomal RNA was translated in the cell-free system. In male rats receiving daily injections of estradiol-17β, preprolactin mRNA activity was increased to an apparent maximum of 300% of controls after 7 days of treatment. Our data also indicate that estradiol increases preprolactin mRNA activity per μg of RNA as well as the pituitary content of RNA. After estradiol treatment was discontinued, preprolactin mRNA activity declined to 50% of the maximum stimulation after approximately 2 days. In ovariectomized retired breeder female rats, a 5-fold increase in preprolactin activity over ovariectomized controls was obtained. In other studies, a 2-fold increase in preprolactin mRNA activity was obtained in male rats 24 h after a single injection of pimozide, a dopamine blocking drug.

Prolactin secretion and synthesis by the pituitary gland is influenced by hypothalamic factors and by estrogens. Estrogen treatment of either males or ovariectomized females results in an increase in the circulating levels, pituitary content, and rate of PRL[1] synthesis (Catt and Moffat, 1967; Neill, 1972; Yamamoto et al., 1976). It is clear that in the female estrogens function in part via the hypothalamus to mediate cyclic changes in PRL release (Neill, 1972). However, several lines of evidence also suggest that estrogens have direct effects on the pituitary. Gersten and Baker (1970) noted that estrogen pellets implanted into one side of the pituitary of ovariectomized rats resulted in hyperplasia and hypertrophy of lactotrophs only on the side ipsilateral to the implant. In experiments in which the pituitary was removed from hypothalamic control by transplantation to the kidney capsule of hypophysectomized rats estrogen treatment resulted in increased serum PRL levels (Lu et al., 1971). Estrogen binding studies using radioautographic techniques (Keffer et al., 1976) or tissue extracts (Leavitt et al., 1969; Notides, 1970) confirm the pituitary as a target tissue for estrogens.

We have recently demonstrated the synthesis of preprolactin in the wheat germ cell-free translation system directed by RNA from rat pituitaries or MtTW10 pituitary tumor tissue (Maurer et al., 1975, 1976). Preprolactin synthesized in the cell-free system shares major tryptic peptide fragments with authentic rat PRL and has a leucine-rich N-terminal addition of 29 amino acids (Maurer et al., 1977). At least two other laboratories have demonstrated a similar translation product with RNA from rate pituitary cell culture lines and in both instances it was shown that thyrotropin releasing hormone increased messenger RNA (mRNA) activity in RNA preparations from these cells (Evans and Rosenfeld, 1976; Dannies and Tashjian, 1976). In this report we present evidence that preprolactin mRNA activity in RNA isolated from the pituitary glands of male or ovariectomized female rats is increased by treatment with either estradiol or pimozide, a dopamine blocking drug.

Materials and Methods

Materials

[^{3}H]Leucine (55–57 Ci/mmol) and NCS tissue solubilizer were obtained from Amersham/Searle (Arlington Heights, Ill.). Optical grade CsCl and RNase-free sucrose were obtained from Schwarz/Mann (Orangeburg, N.Y.). Bio-Gel A_{15} agarose beads were obtained from Bio-Rad Laboratories (Richmond, Calif.) and wheat germ was a gift from General Mills (Vallejo, Calif.).

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; PRL, prolactin; Tris, tris(hydroxymethyl)aminomethane; GH, growth hormone; DEAE, diethylaminoethyl; PBS, phosphate-buffered saline.

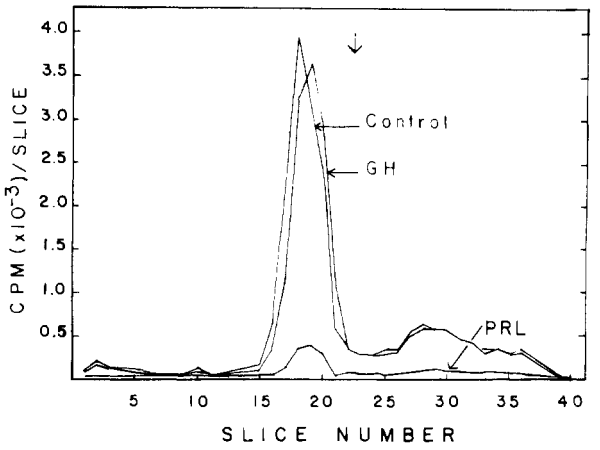

FIGURE 1: Electrophoresis of cell-free translation products adsorbed to solid phase antiprolactin in the presence of either rat PRL or rat GH. Seventy-five-microliter aliquots of a 0.4-mL reaction containing 40.0 μg of rat pituitary RNA were incubated with solid phase antiprolactin with (i) no additions, (ii) 50 μg of NIH RP-1 rat prolactin, or (iii) 50 μg of NIH rat GH. The immunoreaction was carried out at room temperature for 3 h. The solid phase antiprolactin was washed and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Methods. The gels were frozen and cut into 1.6-mm slices, and the radioactivity in each slice was determined. Electrophoresis was from left to right.

Methods

Animals and Injections. Sixty-day-old male, 100–200 g rats (Holtzman Co., Madison, Wis.) were maintained in a temperature-controlled room at least 1 week prior to use. Daily injections of 10 μg of estradiol-17β in 0.1 mL of sesame oil were given subcutaneously. Controls received daily vehicle injections. Animals were sacrificed 24 h after the last injection and anterior pituitaries collected into liquid nitrogen.

RNA Isolation. Frozen pituitaries were homogenized for three 10-s periods in a Tekmar Tissuemizer at 45% of maximum setting in 3.0 mL of 0.1 M Tris-HCl (pH 8.0) containing 4% sodium dodecyl sarcosine (twice recrystallized from ethanol) and 1.0 mg/mL of heparin. RNA from these homogenates was pelleted by centrifugation through a 1.2-mL CsCl cushion as described by Glisen et al. (1974). The upper portion of the resulting supernatant was removed by aspiration. The remaining centimeter of the supernatant (approximately 1 mL) was poured off and the top of the cellulose nitrate tube cut off in order to avoid possible contamination of the pellet with heparin. The drained pellets were dissolved in 0.7 mL of Tris-HCl (pH 7.5) containing 0.5% sodium dodecyl sulfate and 5.0 mM EDTA·Na_2 before adding LiCl to a final concentration of 0.3 M. Two volumes of ethanol was added and RNA allowed to precipitate overnight at −20 °C. RNA was collected by centrifugation and washed three times in 75% ethanol containing 0.2 M NaCl and once with 100% ethanol. The final pellet was dried under vacuum and dissolved in water. poly(A)-RNA was prepared by chromatography on oligo(dT)-cellulose according to the methods of Avin and Leder (1972). RNA concentrations were determined by absorbance at 260 nm and the total RNA extracted by this procedure ranged from 25 to 35 μg/pituitary in nontreated controls which is equivalent to approximately 3 mg of RNA/g of tissue.

Polysomal RNA. Polysomes were prepared from freshly collected anterior pituitaries after homogenization in 4.0 mL of buffer A (25 mM Tris-HCl, pH 7.6, 50 mM KCl, 5.0 mM $MgCl_2$, 1.0 mM dithiothreitol, and 1.0 mg/mL heparin). The homogenate was made 1% in Triton X-100 and centrifuged at 15 000g for 10 min. The supernatant was then layered over a 1.0-mL cushion of 1.0 M sucrose in buffer A and centrifuged for 2 h at 200 000g in a Beckman SW 50.1 rotor. The polysomal pellets were stored in liquid nitrogen until RNA was prepared as described above.

Cell-Free Protein Synthesis. Wheat germ extracts were prepared according to the procedures of Roberts and Paterson (1973) except that the preincubation step was omitted and the extracts were dialyzed for 6–8 h in place of chromatography on Sephadex G-25. Cell-free reactions (100 μL) were prepared as previously described (Maurer et al., 1976) except that the final leucine concentration was 12 μM and 10 μCi of [^{3}H]-leucine (8.3 Ci/mmol) was added to each reaction. Concentrations of Mg^{2+}, K^+, and spermine were 2.0 mM, 75 mM, and 80 μM, respectively. At the end of a 90-min incubation period, the total incorporation into trichloroacetic acid insoluble peptides was determined in 5-μL aliquots. Aliquots were also removed for the determination of the radioactivity in preprolactin (see below).

Immunological Procedures. Sheep were immunized with rat PRL and antibody specificity was determined by immunoprecipitation of [^{3}H]leucine-labeled pituitary extracts as previously described (Maurer et al., 1976). Rat PRL was obtained either as a gift from the National Institute of Arthritis, Metabolic and Digestive Diseases or isolated by the method of Ellis et al. (1969). An antibody-rich fraction was prepared by ammonium sulfate precipitation and DEAE-cellulose chromatography (Kabat and Mayer, 1961). Bio-Gel A_{15} agarose beads were activated by cyanogen bromide and antibodies were covalently attached as previously described (Parikh et al., 1975). The reaction mixture consisted of activated beads added to an equal volume (10 mL) of buffer containing 450 mg of protein. Based on absorbance at 280 nm, reactions were greater than 90% complete. Solid phase antibodies were diluted with phosphate-buffered saline, PBS (0.05 M $NaPO_4$, pH 7.5, 146 mM NaCl, and 0.02% sodium azide), to twice the packed volume. Sheep antisera to rabbit γ-globulin (anti-IgG) was treated identically.

Cell-free reaction products were mixed with solid phase antiprolactin as follows: solid phase antibody (100 μL) and aliquots of cell-free reactions were added to 1.0 mL of PBS containing 2% Triton X-100 with 1% sodium deoxycholate in siliconized tubes. Tubes were then rotated at a 45° angle for either 3 h at room temperature or overnight at 5 °C. Nonreacted products were removed by eight washes at room temperature with PBS–Triton X-100–deoxycholate using low speed centrifugation to sediment the solid phase antibodies between washes. Two subsequent washes in PBS alone were used to transfer the solid phase antibodies to a separate tube in order to avoid radioactivity adsorbed to the surface of the initial reaction tube. The radioactivity incorporated into immunoreactive preprolactin was estimated by one of two methods: (1) in initial experiments the radioactivity migrating as preprolactin on sodium dodecyl sulfate-polyacrylamide gels was quantitated. The washed solid phase prolactin complex was prepared for electrophoresis by adding an equal volume (100 μL) of 0.1 M Tris-HCl (pH 7.4), 8 M urea, 2% 2-mercaptoethanol, and 2% sodium dodecyl sulfate to the final loosely packed pellet. Samples were then heated at 100 °C for 5 min. Electrophoresis and procedures for determining radioactivity in gels were performed as described (Maurer et al., 1976) except that the concentration of bisacrylamide was increased to 0.5%. The radioactivity migrating as preprolactin was quantitated by summing the total peak radioactivity (Figure 1), subtracting a background which was calculated by obtaining the mean radioactivity in three slices on either side of the peak and multiplying this mean by the number of slices in the peak. In the second method, two equal aliquots from a

single cell-free reaction were mixed with solid phase antiprolactin as described above. To determine radioactivity adsorbed nonspecifically to the solid phase antibody, unlabeled PRL was added to one of the reaction tubes in sufficient quantity to maximally compete for the available antibody binding sites. These reactions were always carried out overnight in the cold and the nonadsorbed radioactivity was removed by washing as described above. After the final wash in PBS, 0.8 mL of NCS was added to the pelleted beads followed by vigorous mixing and incubation for 30 min at 37 °C. The mixture was then quantitatively transferred to scintillation vials and the radioactivity determined with an efficiency of 50%. The difference in radioactivity adsorbed to the solid phase antiprolactin in the presence and absence of unlabeled prolactin was taken as a measure of the preprolactin synthesized. In some experiments nonspecific adsorption was estimated by reacting aliquots of cell-free reactions with solid phase anti-IgG.

Miscellaneous Determinations. Pituitary RNA, protein, and DNA were determined as described by Munro and Fleck (1966). Protein concentrations were determined as described by Lowry et al. (1951). Liquid scintillation cocktail contained 5.1 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per L of toluene.

Results

Cell-free products labeled with [^{3}H]leucine that were adsorbed to solid phase antiprolactin and solubilized in sodium dodecyl sulfate–buffer migrated on sodium dodecyl sulfate–polyacrylamide gels predominately as a single peak of radioactivity (Figure 1). The adsorption of preprolactin was specific in that rat PRL, but not rat growth hormone, was effective in reducing the radioactivity migrating as preprolactin (Figure 1). Since reasonably low concentrations of unlabeled antigen were effective in competing for the antibody binding sites on solid phase antiprolactin, quantitative data were obtained to investigate this as a method for determining radioactivity in preprolactin. Aliquots of a cell-free reaction were incubated with solid phase antiprolactin and increasing amounts of unlabeled prolactin. Inclusion of 20–40 μg of prolactin produced maximal reduction in the absorption of radioactivity bound to antiprolactin (data not shown). Similar results were obtained with different preparations of solid phase antibody. Routinely, 30 μg of prolactin was added to the competition assays. The addition of unlabeled prolactin did not affect the fraction of acid-insoluble radioactivity adsorbed to solid phase antiprolactin from cell free reactions directed with RNA from uterus or oviduct (Table I). Moreover, the addition of PRL did not affect the adsorption of radioactivity to solid phase anti-IgG from reactions primed with pituitary RNA. Although the nonspecific adsorption of labeled products to solid phase antibodies was less than that adsorbed to antiprolactin in the presence of PRL, this did not appear to be an effect of unlabeled PRL on nonspecific adsorption.

The [^{3}H]leucine incorporated into preprolactin in response to 10 μg of pituitary RNA as determined by competition with unlabeled PRL was quite reproducible, 5300 ± 350 cpm (mean ± SEM) for three groups of controls and 15 600 ± 630 for three groups of 4-day estradiol treated rats. In this same experiment each RNA sample was translated in duplicate and the amount of radioactivity in preprolactin determined twice in one translation reaction and once in the remaining reaction. Analysis of variance of this data (not shown) indicated that greater than 80% of the variation in this assay was due to variation between animal groups. The adsorption of preprolactin was at least 90% complete following the initial adsorption as determined by mixing the material not adsorbed initially with a second batch of solid phase antiprolactin. Also, the radioactivity in preprolactin increased linearly as the amount of cell-free product increased. Thus, the difference in radioactivity between competed and noncompeted immunoreactions provided a reproducible and convenient method for determining the preprolactin synthesized in response to rat pituitary RNA.

TABLE I: The Influence of PRL on the Immunoreaction between Cell-Free Translation Products and Solid Phase Antibodies.[a]

Experiment	Solid phase antibody	RNA (source)	± PRL	% of radioact. adsorbed
I	PRL	Pituitary	−	33.7
			+	10.1
	PRL	Hen oviduct	−	5.5
			+	6.8
	IgG	Pituitary	−	4.6
			+	4.8
II	PRL	Pituitary	−	30.9
			+	12.3
	PRL	Hen oviduct	−	9.1
			+	8.2
	PRL	Rat uterus	−	9.8
			+	9.5

[a] A 0.5-mL reaction containing 50.6 μg of pituitary RNA (17 900 cpm/5 μL), a 0.4-mL reaction containing 48 μg of hen oviduct RNA (16 000 cpm/5 μL), and a 0.1-mL reaction containing 17.7 μg of immature rat uterine RNA (4900 cpm/5 μL) were run according to procedures outlined in Methods. Forty-five-microliter aliquots of the indicated reactions were mixed and rotated overnight in the cold with either solid phase antiprolactin or globulin in the presence or absence of 30 μg of PRL. The radioactivity reacting with the solid phase antibodies was determined as described in Methods. The cell-free reactions with pituitary or hen oviduct RNA were the same reactions used in experiments I and II except that they were stored frozen between experiments.

Incorporation of [^{3}H]leucine into total acid-insoluble cell-free products and into preprolactin increased linearly with RNA concentration (see Maurer et al., 1976). Also, the fraction of the total radioactivity incorporated into preprolactin was essentially constant with increasing concentrations of RNA. In preliminary experiments it was shown that the optimal concentrations of either magnesium or potassium were the same for incorporation into acid-insoluble proteins and into preprolactin.

When identical amounts of RNA from control, 2- and 4-day estradiol-treated pituitaries were used to direct cell-free translation reactions, the immunoreactive products migrating as preprolactin on sodium dodecyl sulfate–polyacrylamide gels was increased (Figure 2). Although only the portion of the gels containing the preprolactin peak is shown here, in several experiments small increases in the radioactivity migrating ahead of the preprolactin peak were observed in reactions with RNA from estradiol-treated pituitaries.

Response to RNA from estradiol-treated rats as compared with controls was shown to be constant as a function of cell-free incubation times of 20 to 90 min (data not shown).

The cell-free synthesis of preprolactin in response to total RNA, poly(A)-RNA, and polysomal RNA from control and 4-day estradiol-treated rats is shown in Figure 3. Approximately a tenfold increase in the total mRNA activity per unit RNA was obtained in poly(A)-rich RNA compared with total RNA. The preprolactin mRNA activity in both total RNA and poly(A)-RNA from pituitaries of treated rats was 2.5 times greater than the activity in RNA prepared from control rats

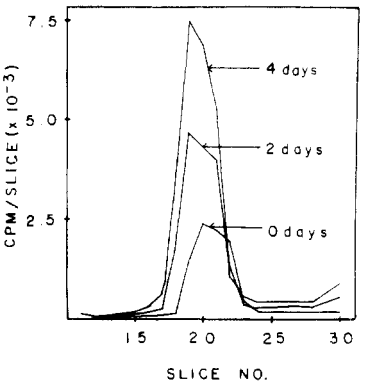

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoreactive cell-free translation products from reactions directed by pituitary RNA from control or estradiol-treated rats. RNA was isolated from rats (4/group) and 10 μg added to 100-μL reactions. Fifty-microliter aliquots were mixed with solid phase antiprolactin, and the nonadsorbed radioactivity was removed as described in Methods. Following electrophoresis of the immunoreacted material, gels were frozen, sliced, and digested in NCS, and the radioactivity in each slice was determined. Only the portions of the gels containing the preprolactin peak are shown.

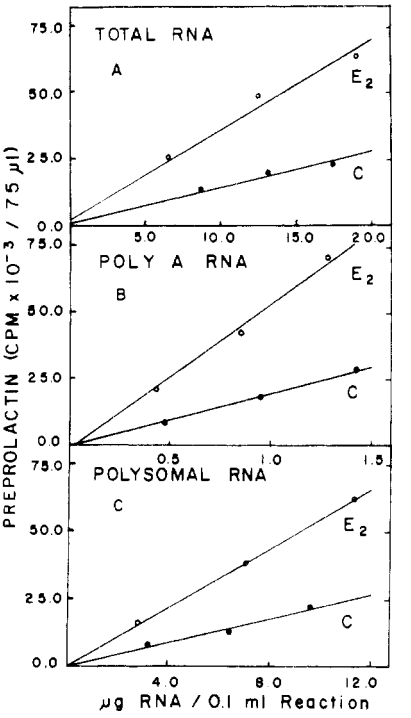

FIGURE 3: The cell-free synthesis of preprolactin in response to total RNA, poly(A)-RNA, or polysomal RNA from control and 4-day estradiol-treated rats. Total RNA from several experiments was pooled and aliquots were withdrawn for translation. Total RNA (260 μg) from either control or treated groups was subjected to chromatography on oligo(dT)-cellulose. The poly(A)-rich RNA eluting in low salt (Avin and Leder, 1972) was concentrated by ethanol precipitation and translated at the concentrations indicated. For both total RNA (A) and poly(A)-RNA (B), the amount of radioactivity incorporated into preprolactin was estimated by mixing 50-μL aliquots of the cell-free reactions with solid phase antiprolactin for 3 h at room temperature and the amount of radioactivity migrating as preprolactin on sodium dodecyl sulfate–polyacrylamide gels determined as described in Methods. A single reaction containing no exogenous RNA was subjected to immunoreaction and electrophoresis to ensure that no peaks of radioactivity comigrating with preprolactin were synthesized from endogenous mRNA (C). Polysomal RNA from groups of ten rats (control and 4-day treated) was prepared as described in Methods. From cell-free reactions containing the indicated concentrations of RNA two 25-μL aliquots were taken for immunoreaction with solid phase antiprolactin. The radioactivity in preprolactin was estimated by subtracting the radioactivity adsorbed in the presence of PRL from that adsorbed in the absence of PRL. In a single reaction containing no exogenous RNA, less than 100 cpm out of 25-μL aliquots was adsorbed to solid phase antiprolactin in the presence and absence of prolactin.

(Figure 3A,B). Polysomes were obtained by centrifugation through a sucrose cushion (Figure 3C) prior to the isolation of RNA by the CsCl procedure (see Methods). The increase in preprolactin mRNA due to estradiol treatment was also 2.5 times greater than controls, suggesting that the estradiol-induced increase in preprolactin mRNA activity observed in preparations of total tissue RNA is not an artifact generated by translation of such an RNA mixture. Also, the results of experiments in which the recovery of globin mRNA activity was estimated or in which pituitaries from control or treated rats were combined prior to RNA isolation indicated that the observed estradiol effect is not due to a general tissue effect that alters the yield or translational quality of the mRNA.

Preprolactin mRNA activity increased slowly with daily injections of estradiol-17β and did not reach an apparent maximum until about 7 days (Figure 4). Little or no increase occurred after 7 days. In previous experiments, preprolactin mRNA activity has been expressed as radioactivity per μg of RNA. This would lead to an underestimate of the overall increase in pituitary preprolactin mRNA activity if estradiol treatment resulted in an increase in pituitary RNA. Daily injections of estradiol increased pituitary weight, protein, DNA, and RNA content (Figure 5) with the increase in RNA clearly the most pronounced. In this same experiment preprolactin mRNA activity was determined in two groups of control and two groups of 4-day treated rats and a 2.9-fold increase in activity per μg RNA was obtained. Since after 4 days of estradiol treatment, the pituitary RNA content was increased to 130% of controls, this suggested that the actual increase in total preprolactin mRNA activity was 3.8-fold. Thus, it would appear that estradiol treatment increases both the concentration of preprolactin mRNA as well as the pituitary RNA content.

Because female rats have a higher basal rate of PRL synthesis than do males, it was of interest to investigate the effect of ovariectomy and subsequent estradiol injections on preprolactin mRNA activity (Figure 6). After 4 days of estradiol treatment ovariectomized females showed a 5-fold stimulation in preprolactin mRNA activity over control, ovariectomized females. This was higher than the 2.5- to 3.0-fold increase obtained in males after the same treatment. Estradiol treatment did not increase preprolactin mRNA activity in intact females.

To determine the effect of estrogen withdrawal on preprolactin mRNA activity male rats were given daily estradiol injections for 7 days before treatment was discontinued (Figure 7). Using a time point of 24 h after the last injection as maximum (100%) stimulation, preprolactin mRNA activity decreased 65% over a 5-day period. No further decrease was noted by 7 days. Although these data are insufficient to be certain of the order by which this response declines, it appears that the response declined by 50% in approximately 2 days.

A single injection of pimozide, a dopamine blocking drug, increased preprolactin mRNA activity to 150% of control values after 12 h and, by 24 h, it had increased to 200% (Figure 8). This effect is apparently maximal by 24 h since no further increase was noted at 48 h.

Discussion

We have used the wheat germ cell-free translation system to show that pituitary preprolactin mRNA activity is increased by estradiol-17β. The adsorption of preprolactin to solid phase antiprolactin proved to be a specific and reproducible method

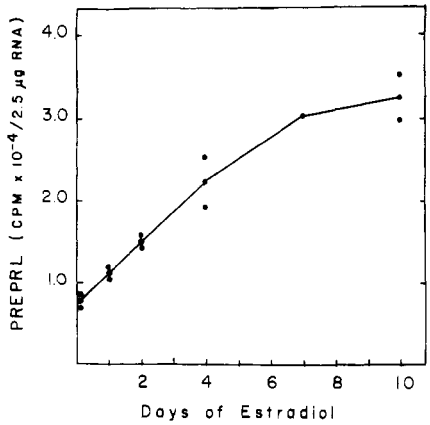

FIGURE 4: The influence of daily injections of estradiol-17β on preprolactin mRNA activity in pituitary RNA. Injections, RNA isolation, and cell-free translations (10 μg of RNA/100 μL of reaction) were as described in Methods. The radioactivity in 25-μL aliquots was determined by competition with prolactin as described in Methods. Individual points represent determinations on duplicate RNA samples from each treatment (three rats/treatment group) and the curve connects mean values (except day 7).

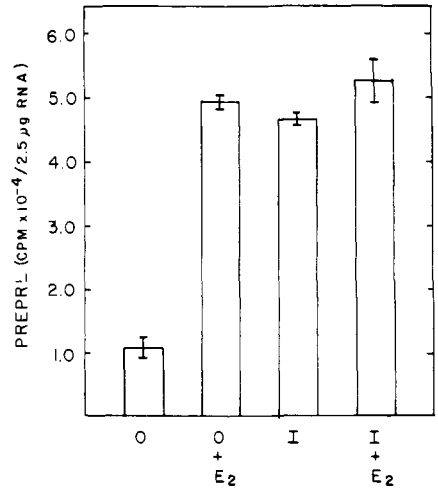

FIGURE 6: The effect of estradiol-17β on preprolactin mRNA activity in ovariectomized and intact female rats. Retired breeder female rats were ovariectomized 10 days prior to the beginning of estradiol injections which were for 4 days. Other procedures were as described in Methods and Figure 4. Values represent the mean ± SEM (n = 3). O, ovariectomies; I, intact; E_2, estradiol-17β.

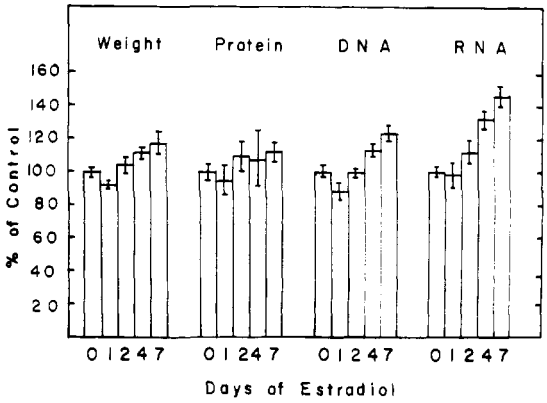

FIGURE 5: The effect of estradiol-17β on pituitary weights, DNA, protein, and RNA content. Rats were given daily injections of estradiol-17β or oil and sacrificed 24 h after the last injection. The indicated determinations were made on each pituitary as described in Methods. Data are expressed as percent of control ± SEM (n = 5).

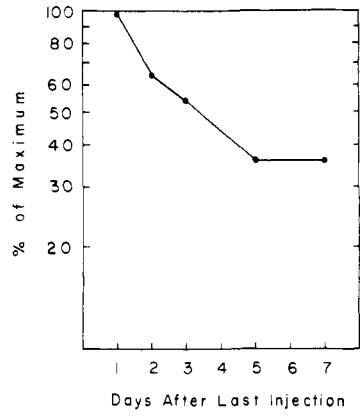

FIGURE 7: The decline in preprolactin mRNA activity after estradiol treatment was discontinued. Male rats were given daily subcutaneous injections of estradiol-17β (10 μg) in saline for 7 days and 24 h after the last injection taken as the zero time (100% stimulation). Injections and withdrawal times were staggered so that all rats were sacrificed at the same time. RNA isolation, cell-free translations, and the estimation of radioactivity in preprolactin were as described in Methods and Figure 4.

for quantitating relative differences in preprolactin mRNA activity. Since the capacity of the solid phase antibody was limited, reasonably low concentrations of unlabeled prolactin were effective in competing with preprolactin for the available antibody binding sites. Rat growth hormone did not compete which is significant as male rat growth hormone accounts for about 20% of the total pituitary protein synthesized (Maurer and Gorski, unpublished observations) and the molecular weight of pregrowth hormone is similar to that of preprolactin (Sussman et al., 1976). The majority of the immunoreactive radioactivity migrated as a single peak on sodium dodecyl sulfate-polyacrylamide gels; however, a portion of the adsorbed radioactivity migrated as a broad peak ahead of preprolactin. Since the wheat germ system is subject to premature termination (Prives et al., 1974) and fragments with a single antigenic determinant would be adsorbed to the solid phase antibody, it is likely that these smaller peptides contain prolactin sequences. Further, the inclusion of unlabeled prolactin in immunoreactions prevented the adsorption of these peptides. Although the fraction of the added radioactivity adsorbed nonspecifically (in the presence of unlabeled PRL) was rather high (5–10%), a statistical analysis indicated that only 9% of the overall variation in the translational assay could be attributed to the immunological part of the procedure. It is possible that solid phase antibodies could be of general use in detecting cell-free translation products especially in instances where antisera with sufficiently high titers to obtain rapid immunoprecipitation are not available.

We have demonstrated that, for equivalent concentrations of pituitary RNA from control and estradiol-treated rats, the total incorporation into acid-insoluble radioactivity was approximately equal while incorporation into preprolactin was increased 2- to 3-fold. Total RNA, poly(A)-RNA, and polysomal RNA were all active in directing the synthesis of preprolactin and the magnitude of the estradiol-induced increase in preprolactin mRNA activity was the same in all three types of RNA preparations. The recovery of mRNA activity was apparently not affected by estradiol treatment since RNA prepared by mixing of control and treated pituitaries prior to homogenization contained the expected amount of preprolactin mRNA activity. Also, the recovery of globin mRNA activity was not markedly different when reticulocyte RNA was added to either control or treated pituitaries prior to RNA isolation. Together these results indicate that the observed increase in

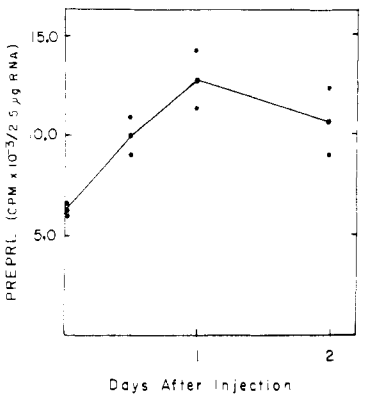

FIGURE 8: The effect of pimozide on preprolactin mRNA activity. Rats were given a single subcutaneous injection of pimozide (0.063 mg/kg in 0.1 M tartaric acid) and sacrified at the times indicated. All other procedures were as described in Methods and Figure 4. Individual points represent determinations from two treatment groups of three rats and the curve connects the mean values.

preprolactin mRNA activity is not artifactual due to estradiol effects on mRNA yield, translational characteristics, or undefined factors contained in preparations of total tissue RNA. Thus, from the available evidence it seems reasonable to conclude that estradiol does indeed increase the pituitary concentration of preprolactin mRNA. Although such conclusions based on data from cell-free translation assays must be carefully interpreted, it is important that in several hormonally dependent systems good agreement has been obtained between relative changes in the synthesis of a particular protein and its cognant mRNA (Rhoads et al., 1973; Shapiro et al., 1973). The time course and magnitude of stimulation of PRL synthesis in male rats following estradiol treatment (Maurer and Gorski, 1977) corresponds quite well with the effects on preprolactin mRNA activity reported here. As the response of both RNA concentration and preprolactin mRNA is relatively slow it is possible that this in part reflects a requirement for lactotroph proliferation before a maximum response is obtained as is the case in some other estrogen-dependent systems (Kohler et al., 1968). Presently it is not possible to determine what fraction of the increase in either total RNA or preprolactin mRNA can be attributed to lactotroph proliferation.

Although our data strongly suggest that estradiol increases pituitary levels of preprolactin mRNA, it is not clear whether this increase is due to an increase in transcription or an increase in mRNA stability. Interpretation is also complicated by the unknown contribution of lactotroph proliferation. If the turnover of the mRNA is slow, it is possible that estradiol could be acting quite early but the relative increase would be too small to be detected in our translation assay (Berlin and Schimke, 1965). Indeed, it was found that in rats in which estrogen treatment was discontinued after 7 days the preprolactin mRNA activity per μ of RNA declined by 50% after about 2 days. Although this is by no means a measure of preprolactin mRNA turnover, it does suggest that it is relatively slow, which could be important in maintaining a high level of pituitary PRL synthesis.

The effect of estradiol on preprolactin mRNA activity in ovariectomized retired breeder female rats was investigated since they present a physiological system that is somewhat different from the males. While these rats were not lactating at the time these experiments were conducted, PRL synthesis accounted for approximately 30% of the pituitary protein synthesized (Stone, unpublished observations). Two weeks after ovariectomy a dramatic decline in preprolactin activity was evident compared with intact controls—an effect that was reversed by estradiol-17β injections. Interestingly, in these ovariectomized rats, 4 days of estradiol treatment resulted in a 5-fold increase in preprolactin mRNA activity. Since this is considerably greater than the 2.5- to 3.0-fold increase usually observed in males after 4 days of treatment, it is possible that the contribution due to lactotroph proliferation is less in rats which have been previously exposed to estradiol.

After a single injection of pimozide, preprolactin mRNA activity was increased to an apparent maximum of 200% of controls in 24 h which is considerably greater than the effect of estradiol 24 h after injection. The increase in preprolactin mRNA is consistent with previous reports that treatment of rats with dopamine blocking drugs increased PRL synthesis (MacLeod and Lehmeyer, 1974). This finding also lends support to the possibility that estrogens may act in part to increase preprolactin mRNA activity by an effect on hypothalamic factors controlling PRL release. Since it is likely that pimozide acts primarily to increase the release of PRL (Ojeda et al., 1974), these data suggest that release of PRL itself may stimulate the production of its mRNA.

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Monosaccharide Transport System of the Human Erythrocyte. Identification of the Cytochalasin B Binding Component†

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ABSTRACT: Sealed, protein-depleted vesicles were formed by treatment of erythrocyte membranes with dimethylmaleic anhydride and isolated by density gradient centrifugation. These vesicles contain the major erythrocyte membrane polypeptides of bands 3, 4.5, 7, and PAS 1-3 but lack those of bands 1, 2, 2.1, 4.1, 4.2, 5, and 6 (nomenclature of Steck, T. L. (1974), *J. Cell. Biol. 62,* 1). The presence of the monosaccharide transport system in the vesicles was demonstrated by the findings that the vesicles transport the substrate L-sorbose and that this uptake is inhibited by cytochalasin B, phloretin, and D-glucose. The activity of the transport system (initial rate of L-sorbose uptake per minute per milligram of membrane protein) is about 95% of that in intact cells. The transport of L-sorbose and the binding of [^{3}H]cytochalasin B to the vesicles are inactivated by reaction of the vesicles with 1-fluoro-2,4-dinitrobenzene. Cytochalasin B protects these functions against inactivation. Differential labeling of the cytochalasin B binding component was accomplished by first treating the vesicles with nonradioactive 1-fluoro-2,4-dinitrobenzene in the absence and presence of cytochalasin B, and then reacting them in the absence of cytochalasin B with tritium-labeled reagent in one case and with ^{14}C-labeled reagent in the other case. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the combined treated vesicles showed a region of selective labeling that coincided with protein band 4.5 (molecular weight range 45 000-65 000). The number of dinitrophenyl groups that can be differentially incorporated, after correction for incomplete protection and partial reaction, is equivalent to about 350 000 per cell. This value agrees approximately with the number of high affinity binding sites for cytochalasin B in the erythrocyte membrane.

The monosaccharide transport system of the human erythrocyte has been extensively investigated with regard to its kinetics, specificity, and susceptibility to inhibition (LeFevre, 1961; LeFevre, 1975; Jung, 1975). Efforts are now underway in a number of laboratories to identify and isolate the components of this transport system (please see the Discussion). Here we report the results of an approach to this problem that relies on the following earlier findings. First, treatment of the erythrocyte membrane with dimethylmaleic anhydride releases most of the peripheral membrane proteins (Steck and Yu, 1973). Second, the erythrocyte membrane and the membrane depleted of peripheral proteins by reaction with dimethylmaleic anhydride possess a set of high-affinity binding sites for cytochalasin B that appear to be a component of the monosaccharide transport system (Lin and Spudich, 1974a,b). Third, N_2ph-F[1] inactivates the transport system in intact cells (Bowyer and Widdas, 1958; Bloch, 1974) and resealed erythrocyte membranes (Jung, 1974), and cytochalasin B protects against this inactivation (Bloch, 1973).

Our rationale has been that identification of the cytochalasin B binding component through differential labeling with N_2ph-F would have the best chance of success if a membrane preparation with a simplified protein composition were used. To this end, we have treated human erythrocyte membranes with dimethylmaleic anhydride to obtain vesicles that lack the peripheral membrane proteins. The monosaccharide transport system was found to be functional in these vesicles. It is inactivated by N_2ph-F, and cytochalasin B protects against this inactivation. These properties have allowed us to identify the cytochalasin B binding component of the transport system through differential labeling with N_2ph-F.

Experimental Procedures

Materials. L-[^{14}C]Sorbose, [^{14}C]N_2ph-F, and [^{3}H]N_2ph-F were purchased from Amersham-Searle. [^{3}H]Cytochalasin B, obtained from New England Nuclear, was purified as described previously (Lienhard and Wardzala, 1976). Freshly outdated units of whole blood or packed cells in citrate-phos-

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[1] Abbreviations used are: N_2ph-F, 1-fluoro-2,4-dinitrobenzene; PAS, periodic acid-Schiff reagent.