Effects of Hypophysectomy and Several Hormone Replacement Therapies upon Patterns of Nucleic Acid and Protein Synthesis and Enzyme Levels in Lactating Rat Mammary Glands

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Abstract

The effects of hypophysectomy and various hormone replacement therapies upon rates of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), casein and cytoplasmic protein synthesis, and enzyme activities in lactating rat mammary glands were studied to gain information regarding the functions of cortisol and prolactin in maintaining lactation. Hypophysectomy resulted in decreases in gland weight as well as RNA and DNA levels and rates of synthesis. Administration of cortisol partially maintained gland weight, RNA and DNA levels, and RNA synthesis but not DNA synthesis. The maintenance of RNA synthesis by cortisol appeared to be specific in that only the synthesis of one of the two RNA fractions studied was maintained. Deoxyribonucleic acid levels and rates of synthesis were maintained by administration of prolactin which also partially maintained gland weights. Administration of prolactin and cortisol together resulted in the maintenance of apparently normal gland weights and RNA and DNA levels and rates of synthesis. Rates of casein and cytoplasmic protein synthesis were decreased by hypophysectomy, partially maintained by cortisol, and apparently maintained at normal levels by administration of prolactin and cortisol plus prolactin. Enzyme levels were reduced in hypophysectomized rats receiving oxytocin alone, oxytocin plus cortisol, or oxytocin plus prolactin. The levels of several enzymes, most notably glucose-6-P dehydrogenase, were maintained by treatment with cortisol plus prolactin, while the activities of several others were not maintained.

Most anatomical and physiological studies indicate that the minimal hormonal requirements for maintaining lactation in rodents are prolactin and cortisol (3, 8, 12). However, few data are available concerning specific hormonal effects associated with maintaining secretory activity. The study of the effects of hypophysectomy and various hormone replacement therapies upon rates of DNA, RNA, casein and cytoplasmic protein synthesis, and upon enzyme activities in rat mammary glands was undertaken to gain information on the functions of cortisol and prolactin in the maintenance of lactation.

Materials and Methods

Animals. Primiparous Sprague-Dawley rats hypophysectomized or sham operated on the tenth day of lactation were purchased with their litters from Berkeley Pacific or Simenson Laboratories and delivered within 24 hours of hypophysectomy. The rats were maintained on Purina Laboratory Chow, sugar cubes, and water with 5% dextrose added for 2.5 days while the several hormonal treatments were administered. At the end of the treatment period, the litters were removed and 0.35 mc of carrier-free 32P and 6 mc of 14C leucine were injected intraperitoneally. One hour after the administration of the isotopes, the mothers were killed by decapitation. Their mammarys were quickly removed and homogenized. Each rat was examined after sacrifice for residual pituitary tissue, and mammary samples from incompletely hypophysectomized animals were discarded.

Four hormone treatments were used. The hypophysectomized rats were injected subcutaneously four times daily with either 0.5 unit of oxytocin (O), 0.5 unit of oxytocin plus 0.25 mg hydrocortisone acetate (CO), 0.5 unit oxytocin plus ten units prolactin (PO), or 0.5 unit of oxytocin plus 0.25 mg hydrocortisone acetate plus ten units prolactin (CPO). Oxytocin and prolactin were dissolved in isotonic saline for injection, and cortisol was prepared and injected separately in isotonic saline containing Tween-80.

Mammary glands were homogenized immediately after collection in four volumes of ice-cold 0.4 M KCl with a glass-teflon homogenizer and strained through two layers of cheese cloth to remove tissue fragments. Aliquots for RNA fractionation and nuclei isolation were removed.
immediately and these separations initiated. The remaining portion of the homogenate was dispersed into three separate tubes and stored frozen for a few days until used for total RNA and DNA analysis, enzyme determinations, and preparation of casein and extractable protein.

Isolation of nuclei. The aliquot of homogenate, usually 20 ml, removed for isolation of nuclei was mixed with an equal volume of 0.25 M sucrose containing 0.004 M CaCl₂, 0.002 M Mg acetate, and 0.01 M tris-HCl, pH 7.6, and centrifuged at 4°C and 3000 × g for 20 minutes. The nuclei were resuspended in 5.0 ml of 0.25 M sucrose containing 0.002 M CaCl₂, 0.001 M Mg acetate, and 0.005 M tris-HCl, pH 7.6, and layered over 25 ml of 0.75 M sucrose containing the same salts. This step was repeated once. The nuclei were then resuspended and washed twice in 0.14 M KCl. The final preparation was resuspended in 5.0 ml of 0.14 M KCl and used for analysis of nuclear RNA-³²P and DNA-³²P.

Isolation of RNA fractions. Fractionations of RNA were done according to modifications of the differential extraction procedures described by Wicks et al. (17), Greenman et al. (4), and Tsanev et al. (16). The aliquot of homogenate, usually 20 ml, removed immediately after preparation for RNA fractionation, was mixed with two volumes of 80% phenol, pH 6.0, and one volume of 0.14 M KCl containing 2 mg/ml of purified bentonite and 1% sodium deoxycholate. The mixture was stirred slowly at 23°C for 30 minutes and then centrifuged at 4°C and 20,000 × g for 5 min. The phenol layer was discarded and the aqueous layer was retained at 0°C. The pellet and interface layers were extracted again at 23°C with equal volumes of 80% phenol, pH 6.0, and 0.14 M NaCl containing deoxycholate and bentonite. After centrifugation, the phenol layer was discarded and the aqueous fraction combined with that obtained in the first extraction. This fraction is referred to as Fraction 1. The residue and interface layers resulting from the extractions at 23°C were extracted twice more at 75°C for 5 min with equal volumes of 80% phenol, pH 6.0, and 0.14 NaCl containing 0.5% sodium lauryl sulfate. These aqueous portions were combined and identified as Fraction 2.

Fractions 1 and 2 were purified essentially as described by Wicks et al. (17) and Greenman et al. (4). Both fractions were deproteinized further by adding 0.5% sodium lauryl sulfate and extracting twice with equal volumes of phenol. The phenol extracts were removed by centrifugation. Ribonucleic acid was precipitated from the final aqueous phase after addition of 2.0% potassium acetate by adding two volumes of 100% ethanol and standing overnight at −15°C. The precipitates were recovered by centrifugation, washed twice with 70% ethanol, and dissolved in 5.0 ml of 0.01 M tris, pH 7.6, containing 0.001 M MgCl₂. This solution was extracted twice with equal volumes of ether and the residual ether removed by bubbling N₂ through the solution. Small molecular weight transfer-type RNA was removed from Fraction 1 by precipitating the nontransfer RNA with 1 M NaCl and twice reprecipitating this RNA with 1 M NaCl after resuspension in tris buffer containing MgCl₂.

Both Fractions 1 and 2 were contaminated with protein and DNA. Therefore, the amounts, specific activities, and base compositions of RNA's in these fractions were determined after precipitation, and one washing with 0.2 × perchloric acid and hydrolysis in 0.3 M KOH for 18 to 20 hours (11). Fraction 1 represented the bulk of the extractable RNA with yields after fractionation of 50-70% of the total RNA. The nucleotide composition of this fraction is presented in Table 1. Based upon the yield, method of extraction, and base composition, Fraction 1 was assumed to consist primarily of ribosomal and ribosomal precursor RNA. Fraction 2 represented 3-6% of the total RNA and had a nucleotide ratio of about 1.0 (Table 1). This fraction was considered to be roughly equivalent to the DNA-like RNA isolated by Greenman et al. (4).

Chemicals and analytical methods. Enzyme assays and auxiliary determinations were done as described previously (1). Radioassays were done in a liquid scintillation counter as described previously (2). Corrections of observed counts were calculated according to the channel ratios technique. The incorporation of ³²P into RNA is dependent upon the specific activity of the tissue precursor pool of ³²P (4, 5) as well as upon rate of synthesis of nucleic acids. In our study, we found that the specific activities of the tissue ³²P varied from animal to animal. Therefore, it was considered necessary to standardize estimates of incorporation of

<table>
<thead>
<tr>
<th>Bases</th>
<th>A + U</th>
<th>G + C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction Ade- Ur- Gu- Cyto- A + U/</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine dine nine nine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mole % 24.0</td>
<td>25.0</td>
<td>26.0</td>
</tr>
<tr>
<td>³²P % 24.2</td>
<td>22.3</td>
<td>22.4</td>
</tr>
<tr>
<td>2 mole % 25.4</td>
<td>23.5</td>
<td>19.8</td>
</tr>
<tr>
<td>³²P % 26.6</td>
<td>26.1</td>
<td>33.2</td>
</tr>
</tbody>
</table>

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radioactivity in the nucleic acids by multiplying the observed incorporation data by a factor to correct for differences in tissue \(^{32}\)P specific activities. This factor was determined by dividing the specific activity of the tissue inorganic \(^{32}\)P in individual animals by the average specific activity of inorganic \(^{32}\)P in all animals. Data thus standardized were more representative of the rate of nucleic acid synthesis and were less variable. Chemicals, reagents, and hormones were purchased commercially with the exception that bovine prolactin (NIH-P-B-2) was supplied by the Endocrinology Study Section, National Institutes of Health, Bethesda, Maryland.

Casein was precipitated from centrifuged (20,000 \(\times\) g; 15 min) homogenates with rennin (15) and purified by repeated isoelectric precipitations to constant specific activity. Extractable proteins were precipitated from homogenates with ammonium sulfate (0-60% saturation) after removal of casein and were counted after twice resuspending the precipitate in 0.14 \(\times\) KCl and precipitating again with ammonium sulfate (2). Ribonucleic acid and deoxyribonucleic acid determinations in homogenates and in nuclei preparations were done essentially as recommended by Munro and Fleck (11) and Santen and Arganoff (13) with the following modifications: Two periods of hydrolysis of RNA with 0.3 \(\times\) KOH at 37 C were done. The first hydrolysate, obtained after one hour of hydrolysis, contained from 95-98% of the RNA and was used for estimation of RNA levels in tissue and nuclei. The second hydrolysis period lasted 18 to 20 hours and was carried out to remove residual RNA, and thereby prevent contamination of the DNA hydrolysate which might have introduced error in the determination of DNA specific activities. Nucleic acid precipitations and washes were done in 0.2 N HClO\(_4\) at 0 C. Deoxyribonucleic acid hydrolysis was done at 70 C for 60 minutes in 0.2 \(\times\) HClO\(_4\). Nucleotide analyses were done by thin layer chromatography on polyethyleneimine (PEI) cellulose (14).

**Results and Discussion**

Results reflecting the general nature of the responses to the hormone treatments are presented in Table 2. The pups lost considerable weight in the oxytocin, oxytocin plus cortisol, and oxytocin plus prolactin treatment groups, indicating that these treatments failed to maintain lactation. Pup performance was considerably better in the oxytocin plus cortisol plus prolactin treatment group, but was still much better than that observed in the pups of the sham operated controls, indicating that this hormone treatment supported only partial maintenance of lactation. These results agree with previous reports (3, 7, 12). Treatment with cortisol and prolactin together maintained mammary gland weight, RNA, and DNA at close to normal levels. Prolactin plus oxytocin appeared to maintain normal total gland RNA and DNA levels. Some decrease in gland weight was apparent in the prolactin treatment group, although this effect may have been due to a decrease in milk in the tissue, since RNA and DNA per gram tissue were high compared to the sham operated controls. Unfortunately, it was not possible to determine amounts of retained milk in the samples, because of a lack of sufficient tissue. Treatment with cortisol and oxytocin appeared to support some maintenance of gland weight and total RNA and DNA levels as compared to treatment with oxytocin alone, but regressions of gland size and nucleic acid content are evident in both of these treatment groups. This failure to maintain gland size and nucleic acid content is consistent with the failure of these treatment groups to maintain lactation.

The activities of several enzymes, selected on the basis of previous studies (1, 2), were determined to assess possible metabolic effects of the treatments. The effects of hypophysectomy and the hormone replacement therapies are compared in Table 3. Several noteworthy responses were observed. The activities of the first three enzymes reported in Table 3 were maintained at approximately the same levels in the cortisol plus prolactin treatment group as compared to the sham operated controls, while the activities of the last three enzymes listed in the table were depressed in the group.

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**Table 2. General characteristics of experimental animals.**

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>Body wt (g)</th>
<th>Change in pup wt (avg)</th>
<th>Mammary gland wt (mg/g tissue)</th>
<th>Mammary gland RNA (mg/g tissue)</th>
<th>Mammary gland DNA (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>390</td>
<td>-1.6</td>
<td>5.3</td>
<td>3.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Cortisol*</td>
<td>397</td>
<td>-1.7</td>
<td>7.8</td>
<td>3.7</td>
<td>1.4</td>
</tr>
<tr>
<td>Prolactin*</td>
<td>416</td>
<td>-1.3</td>
<td>8.9</td>
<td>5.1</td>
<td>1.8</td>
</tr>
<tr>
<td>Cortisol + prolactin*</td>
<td>366</td>
<td>-0.3</td>
<td>10.6</td>
<td>4.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Sham*</td>
<td>367</td>
<td>+1.3</td>
<td>12.2</td>
<td>4.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Hypophysectomized on tenth day of lactation and treated with hormone(s) indicated, plus oxytocin four times daily for three days.

Sham operated controls.
of hypophysectomized animals receiving cortisol plus prolactin. The significance of this observation is not known and a number of possibilities including actions by hormones not examined in the present study must be considered. The activities of P-glucose mutase, aldolase, P-fructose kinase, and malate dehydrogenase in the hypophysectomized animals did not appear to be affected significantly by the hormone treatments. The activity of 6-P-glucose dehydrogenase appeared to be maintained at slightly higher levels by the prolactin and cortisol plus prolactin treatments than by the other treatments. The activity of glucose-6-P dehydrogenase was markedly higher in the group treated with cortisol plus prolactin than in the other groups. This latter observation seems important, since it may represent an exception to a previously posed generalization that the activities of many enzymes in mammary tissue reflect an inherent property of secretory cells. The maintenance of the activity of glucose-6-P dehydrogenase expressed on a total gland basis, a per gram tissue basis, or a per milligram DNA basis appeared to be specifically enhanced by treatment with cortisol plus prolactin. Neither hormone was as effective when administered alone.

The amounts of 32P incorporated into DNA are presented in Table 4. The data indicate a higher relative rate of synthesis of DNA in hypophysectomized rats treated with prolactin than in those not treated, suggesting that prolactin may be involved in some manner in the regulation of DNA synthesis and cell proliferation. In addition, it can be suggested that the failure of the hypophysectomized animals treated with oxytocin alone or cortisol and oxytocin to maintain gland size was due to an insufficiency in rate of cell proliferation. These suggestions are consistent with previous reports that prolactin stimulates secretory cell proliferation as well as secretory activity (7, 8, 12); however, they are inconsistent with a recent report that prolactin affects secretory activity but not cell proliferation in vitro in mammary explants from pregnant mice (6). In view of this discrepancy, both direct and indirect mechanisms of prolactin action in increasing rates of DNA synthesis in the present study must be considered. An indirect mechanism involving a general metabolic change might affect mammary DNA synthesis directly, or through alterations in the secretion of hormones such as insulin. The luteolytic action of prolactin (9) might be implicated as an indirect mechanism in view of the known functions of ovarian hormones in lactation. There is also a possibility that the apparent stimulation of DNA synthesis was due to a higher rate of cell

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**Table 3. Activities of several enzymes in mammary glands.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>None</th>
<th>Cortisol</th>
<th>Prolactin</th>
<th>Cortisol + prolactin</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} )</td>
<td>( s_{\bar{x}} )</td>
<td>( \bar{x} )</td>
<td>( s_{\bar{x}} )</td>
<td>( \bar{x} )</td>
</tr>
<tr>
<td>Glucose-6-P dehydrogenase</td>
<td>0.76</td>
<td>0.18</td>
<td>1.32</td>
<td>0.20</td>
<td>3.55</td>
</tr>
<tr>
<td>6-P-glucose dehydrogenase</td>
<td>0.28</td>
<td>0.06</td>
<td>0.34</td>
<td>0.16</td>
<td>0.58</td>
</tr>
<tr>
<td>P-glucose mutase</td>
<td>0.31</td>
<td>0.08</td>
<td>0.36</td>
<td>0.18</td>
<td>0.47</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphatase</td>
<td>0.12</td>
<td>0.04</td>
<td>0.11</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>P-fructose kinase</td>
<td>0.41</td>
<td>0.03</td>
<td>0.38</td>
<td>0.12</td>
<td>0.48</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>2.47</td>
<td>0.39</td>
<td>4.45</td>
<td>0.88</td>
<td>4.15</td>
</tr>
</tbody>
</table>

- Treatments as described in text and legend of Table 2. Means \( \bar{x} \) and standard errors of means \( s_{\bar{x}} \; N = 6 \) presented as moles substrate converted per minute per gland.

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**Table 4. Incorporation of 32P into nucleic acids.**

<table>
<thead>
<tr>
<th>Nucleic acid fractions</th>
<th>None</th>
<th>Cortisol</th>
<th>Prolactin</th>
<th>Cortisol + prolactin</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0.31</td>
<td>0.41</td>
<td>0.67</td>
<td>0.93</td>
<td>0.82</td>
</tr>
<tr>
<td>Nuclear RNA</td>
<td>14.6</td>
<td>15.0</td>
<td>23.4</td>
<td>27.3</td>
<td>13.0</td>
</tr>
<tr>
<td>RNA Fraction 1</td>
<td>0.05</td>
<td>0.05</td>
<td>0.12</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>RNA Fraction 2</td>
<td>0.13</td>
<td>0.29</td>
<td>0.38</td>
<td>0.65</td>
<td>0.75</td>
</tr>
</tbody>
</table>

- Treatments as described in text and legend of Table 2. Data expressed as specific activities calculated as count/min \( \times 10^9 \)/mg nucleic acid. Different superscripts indicate statistically significant differences \( P > 0.05 \).
TABLE 5. Incorporation of leucine-3H into cytoplasmic protein and casein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>None</th>
<th>Cortisol</th>
<th>Prolactin</th>
<th>Cortisol + prolactin</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$</td>
<td>$s_x$</td>
<td>$\bar{x}$</td>
<td>$s_x$</td>
<td>$\bar{x}$</td>
</tr>
<tr>
<td>Cytoplasmic protein</td>
<td>1.6</td>
<td>0.35</td>
<td>3.1</td>
<td>0.61</td>
<td>5.0</td>
</tr>
<tr>
<td>Casein</td>
<td>3.8</td>
<td>1.38</td>
<td>11.5</td>
<td>0.07</td>
<td>19.6</td>
</tr>
</tbody>
</table>

*Treatments as described in text and legend of Table 2. Means ($\bar{x}$) and standard errors of means ($s_x$; $N = 4$) presented as count/min $\times 10^{-6}$/g tissue.*

turnover resulting from increased secretory activity in prolactin-treated animals. However, if this were the case one would expect greater differences in DNA synthesis between the prolactin and prolactin plus cortisol treated groups, and between the prolactin plus cortisol group and the sham operated controls, since pup growth and, presumably, secretory activities were different in these groups. Although previous data (7, 10) strongly indicate that prolactin acts at the level of the mammary gland in stimulating cell proliferation and our data appear to support this conclusion, there seems to be sufficient doubt raised by other work to consider the question unresolved.

The incorporation of $^{32}P$ into several fractions of RNA was investigated in an attempt to gain insight relative to the nature of the hormonal effects reflected by the treatment differences in RNA level indicated in Table 1. The specific activity of nuclear RNA was not changed from normal by hypophysectomy or treatment with cortisol and was above normal in hypophysectomized animals treated with prolactin and prolactin plus cortisol. Lack of an effect of hypophysectomy upon the relative rate of nuclear RNA synthesis may have been due to the presence of greater quantities of RNA in nuclei from sham operated animals, since the RNA/DNA ratio was always higher in nuclei from these animals. However, the nuclei obtained in the present study were not considered sufficiently pure to support this suggestion. The relative rates of synthesis of RNA's in Fraction 1 were depressed by hypophysectomy and were restored to essentially normal levels when prolactin was administered. Cortisol did not affect the relative or absolute rates of synthesis of RNA's in Fraction 1. The synthesis of RNA's in Fraction 2 were depressed by hypophysectomy, partially restored when either cortisol or prolactin was administered, and fully restored when cortisol and prolactin were administered. That cortisol maintained the synthesis of RNA's in Fraction 2 but not in Fraction 1 indicates that cortisol acts with specificity in effecting RNA synthesis. Prolactin appeared to act in a more general fashion, effecting the synthesis of RNA's in both Fractions 1 and 2. The possibility that prolactin acts with specificity is not excluded.

Cytoplasmic (extractable) protein synthesis and casein synthesis were investigated as criteria reflecting gland maintenance and function, respectively. The data, presented in Table 5, indicate that hypophysectomy reduces both cytoplasmic protein and casein synthesis. Cortisol appeared to partially maintain protein synthesis. It was surprising to find that casein synthesis was maintained at approximately normal rates in the prolactin and prolactin plus cortisol treatment groups. Based on the growth of the pups of animals in these treatment groups as compared to the sham operated controls, one might have expected a reduction in casein synthesis.

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