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Reduced Prolactin Binding to Liver Membranes During Pheromonal Emission in the Rat

THERESA M. LEE, BERNARD HALPERN, CHUNG LEE AND HOWARD MOLTZ[1]

Committee on Biopsychology, The University of Chicago, IL 60637
Departments of Urology and Obstetrics and Gynecology
Northwestern University Medical School, Chicago, IL 60637

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LEE, T. M., B. HALPERN, C. LEE AND H. MOLTZ. *Reduced prolactin binding to liver membranes during pheromonal emission in the rat.* PHARMAC. BIOCHEM. BEHAV. 17(6) 1149–1154, 1982.—Between 14 and 27 days of lactation, female rats excrete a pheromone in their feces that is cholic-acid dependent and that strongly attracts young. Previous research has shown that high circulating levels of prolactin are necessary before the pheromone can be emitted. However, during the time of pheromonal emission prolactin in serum conspicuously declines, while in hepatic cytosol the hormone reaches peak levels. We were interested in the question of how the liver can show peak cytosolic concentrations of prolactin at a time of falling blood levels of prolactin. Accordingly, we examined the prolactin binding capacity of liver membrane fractions during selected periods of lactation. We also studied the livers of virgin and pregnant females for comparison. Three membrane fractions were separated: the cell membrane, the nuclear membrane and a fraction consisting of the cell membrane and large non-nuclear organelles. In all three fractions, there was an increase in available and total prolactin binding in the liver when pregnant females were compared with nulliparous females. However, during the time of pheromonal emission, when prolactin in hepatic cytosol was elevated, there was a significant reduction in the prolactin binding capacity of the liver. How such a reduction increases the cytosolic concentration of the hormone and in turn heightens cholic acid output and pheromonal emission remains unsolved.

Prolactin Liver membranes Maternal pheromone

IT IS known that lactating rats, between 14 and 27 days postpartum, carry in their feces a pheromone that strongly attracts young [22,23]. This pheromone is synthesized in the cecum and is dependent on heightened levels of cholic acid, a primary liver steroid [17,18]. That heightened cholic acid is necessary for the appearance of the pheromone was established by demonstrating (a) that cholic acid is significantly elevated in bile during the period of pheromonal emission [18], and (b) that increasing the biliary concentration of cholic acid induces the pheromone in otherwise non-pheromone emitting females [17].

Prolactin appears also to be involved in pheromonal emission. For example, ergocornine hydrogen maleate, a dopamine agonist, blocks the pheromone in lactating rats [21] and exogenous prolactin alone elicits the pheromone in nulliparous rats [16,20].

Moltz and Lee [26] attempted to explain how prolactin and cholic acid interact to promote pheromonal emission. Briefly, they suggested that a critical prolactin concentration is reached in the maternal liver beginning about 14 days postpartum, which in turn leads to an above-normal output of cholic acid. When cholic acid is thus heightened, some fraction is thought to escape the enterohepatic circulation, reach the cecum and become metabolized through the action of cecal bacteria. As pictured by Moltz and Lee, the pheromone excreted by the lactating female is either some unidentified derivative of cholic acid or some unidentified end-product arising from cholic-induced changes in the metabolic activity of gut bacteria.

A problem with the explanation just advanced is that heightened serum values of prolactin are not sustained throughout lactation, but are witnessed only during the first 10–12 days postpartum [1,25]. Thereafter, that is in the ensuing pheromonal period, the hormone in blood conspicuously declines. The question is how can the pheromone be prolactin dependent since it is emitted at a time of falling prolactin levels? Lee, Lee and Moltz [19] suggested that the answer might be found by looking, not in blood, but in hepatic tissue. What they showed was that although serum concentrations of prolactin indeed decrease during the period of pheromonal emission, hepatic intracellular levels of the hormone are significantly elevated [19].

[1]Requests for reprints should be addressed to Dr. Howard Moltz, Committee on Biopsychology, The University of Chicago, Chicago, IL 60637.

Although our knowledge of the action of prolactin in hepatic cells is incomplete, it is widely accepted that circulating prolactin first binds to specific receptors on the cell surface [29]. Then, through a pinocytotic-like process [31,32], the ligand-receptor complex enters the cell and becomes bound to such organelles as Golgi and lysosomes [12]. It is not yet clear whether prolactin also binds to nuclear membranes in the liver, although such binding has been demonstrated in other prolactin-sensitive tissues [5,27]. One result may be an alteration of gene expression. With reference to the pheromone, we think of a critical concentration of cytosolic prolactin as inducing selected enzymatic changes in the liver which lead to an increase in the synthesis of cholic acid.

But this raises another question: how can the liver in turn show peak cytosolic concentrations of prolactin at a time of falling blood-levels of the hormone? Since prolactin induces its own hepatic receptors [7, 14, 24, 28, 30], we thought the elevation of the hormone during the first 10–12 days postpartum would leave an elevated number of binding sites in evidence during the ensuing pheromonal period. Such a heightening of receptor capacity, if it in fact occurred, might explain the high cytosolic concentrations of prolactin under decreasing serum concentrations of the hormone. Accordingly, we measured the specific binding of radiolabelled prolactin in various membrane fractions of rat liver expecting to find elevated receptors in at least one cellular compartment. What we found was contrary to expectation.

METHOD

Animals

A total of 84 Wistar rats, reared at The University of Chicago, were selected from the following groups: virgin females in diestrus, pregnant females 18 days post coitus and lactating females either 1, 5, 12, 21, or 30 days postpartum. Each postpartum female was allowed to give birth routinely and subsequently care for a litter of six young. At the time of sacrifice, an animal was removed from the colony room and quickly decapitated. Serum specimens were collected and frozen for later prolactin assay. The liver was rapidly excised, weighed and immediately processed for analysis.

Preparation of Liver Membranes

After removal, the liver from 6 animals in each group was placed in an ice-cold beaker containing 100 ml of cold Buffer A (1 mM sodium bicarbonate, 10 mM calcium chloride, pH 7.0). The tissue was minced and homogenized by a Polytron PT 10 (Brinkman) homogenizer for 30 sec, and for an additional 15–20 sec when necessary. The homogenate was diluted to 600 ml with chilled Buffer A and centrifuged at 900×g for 20 min at 4°C. The resulting precipitate was discarded. The supernatant fraction was then centrifuged at 20,000×g for 20 min at 4°C. The precipitate was designated as the cell membrane (plasmalemma) fraction [4,6]. The livers of the remaining six animals from each group were collected and homogenized in the same fashion. The homogenate was diluted to 300 ml with chilled Buffer A and centrifuged at 150×g for 20 min at 4°C. The resulting precipitate was designated as the fraction of nuclear membranes. The supernatant fraction was then centrifuged at 20,000×g for 20 min at 4°C. The precipitate was designated as plasmalemma plus non-nuclear large organelles (e.g., Golgi, lysosomes, mitochondria) [7,35]. The above three membrane preparations were separately washed with 10 ml ice-cold Buffer B (0.1% bovine serum albumin in 25 mM sodium phosphate and 0.5 mM magnesium chloride, pH 7.0). Each washed fraction was resuspended in 8 ml Buffer B. Aliquots of 1 ml in volume were stored at −80°C for later use for the study of prolactin binding.

Preparation of Radioiodinated Prolactin

Iodination of ovine prolactin was prepared by the method of Thorell and Johanson [36] with lactoperoxidase as modified by Shiu, Kelly and Friesen [34]. Ovine prolactin (4 μg in 20 μl of 25 mM sodium phosphate buffer, pH 7.0), lactoperoxidase (5 μg in 10 μl of 25 mM sodium phosphate buffer, pH 7.0) and ^{125}I (1.25 mCi in 12.5 μl sodium hydroxide, Amersham) were added to a 10×75 mm borosilicate glass tube. The reaction was initiated by adding 10 μl of 22 mM hydrogen peroxide. After 3 min, an additional 10 μl of hydrogen peroxide was added. After an additional 3 min, the reaction was quenched by the addition of 438 ml Tris-BSA buffer (0.1% bovine serum albumin in 25 mM Tris-HCl, pH 7.0). The iodinated prolactin was passed through a Sephadex G-100 column (1×30 cm, Pharmacia), equilibrated with the Tris-BSA buffer [2]. The column was eluted at room temperature with Tris-BSA buffer and 60 fractions of approximately 0.25 ml (5 drops) were collected into tubes coated with 0.1 ml Tris-BSA. The fractions containing high specific activity were pooled and diluted with Tris-BSA buffer so that an aliquot of 0.1 ml contained 90,000 cpm. The specific activity of the iodinated prolactin was determined by precipitating 10 μl of the eluate in 1.5 ml of 10% BSA with 2 ml of 10% trichloroacetic acid and counting for ^{125}I in the precipitate. The specific activity of ^{125}I-prolactin was 130 μCi/μg which was calculated according to the procedure of Shiu, Kelly and Friesen [34].

Prolactin Binding Assay

Prior to incubation with ^{125}I-prolactin, all membrane fractions (plasma membrane, nuclear membrane and plasma plus large organelle membranes) were adjusted to a concentration of 150 μg/100 μl of protein in Buffer B for receptor determinations. When sufficient membrane was not available, a concentration of 100 μg/100 μl was used. An aliquot of 100 μl of the membrane preparation was added to a 12×75 mm polyethylene test tube. All samples were run in quadruplicate. Two tubes with 100 μl of membrane were washed with 0.3 ml 5 M $MgCl_2$ for 15 minutes as per the method of Van der Gugten *et al.* [37]. This procedure was able to remove the prolactin from the receptor sites without destroying the binding capacity of the receptor [13]. Approximately 40% of the protein was lost as a result of the $MgCl_2$ wash, and the final determination of capacity receptors was adjusted accordingly. To the membrane preparation was added specific amounts of unlabelled prolactin in 200 μl of Tris-BSA buffer. This was allowed to incubate at room temperature for 20 min before the addition of 90,000 cpm/100 μl of ^{125}I-prolactin in Tris-BSA buffer.

Six levels of unlabelled prolactin were selected: 0, 0.1, 1.0, 5.0, 10, 50 and 100 ng/ml. We found that 100 ng/ml maximally displaced ^{125}I-prolactin. Incubation was carried out at 23°C for 20 hr. Following the incubation, all tubes were diluted with 2 ml cold 10 mM sodium phosphate and were centrifuged at 4,000×g for 30 min. The supernatant fractions were discarded, and the precipitates were counted for radioactivity. The amount of total ^{125}I-prolactin bound to

TABLE 1

AVERAGE OF THE TOTAL PROLACTIN RECEPTOR CAPACITY IN MEMBRANE PREPARATIONS OF THE LIVER IN VIRGIN, PREGNANT AND LACTATING RATS

	femtomoles/mg protein					
	Plasmalemma		Plasmalemma + Large Organelle Membranes		Nuclear Membrane	
Animals	Unwashed	$MgCl_2$ Washed	Unwashed	$MgCl_2$ Washed	Unwashed	$MgCl_2$ Washed
Virgin-diestrus	131.0 ± 50.9	146.6 ± 21.6	114.1 ± 86.0	112.0 ± 83.3	70.4 ± 67.6	86.0 ± 45.1
Day 18 gestation	146.1 ± 45.8	358.8 ± 133.2	217.4 ± 59.3	483.4 ± 102.1	160.9 ± 48.3	247.4 ± 71.2
Day 1 lactation	149.6 ± 130.2	159.0 ± 130.7	137.6 ± 70.9	262.2 ± 87.4	88.5 ± 14.2	141.1 ± 12.7
Day 5 lactation	63.9 ± 23.1	139.4 ± 77.3	121.2 ± 50.6	136.5 ± 22.0	158.2(n=1)	73.3 ± 40.1
Day 12 lactation	77.4 ± 32.2	52.0 ± 38.9	96.8 ± 45.4	94.4 ± 39.8	24.6 ± 17.8	132.4 ± 87.4
Day 21 lactation	42.3(n=1)	55.7 ± 33.3	41.0 ± 14.9	74.7 ± 33.2	0	17.8(n=1)
Day 30 lactation	30.3 ± 11.8	60.5 ± 4.4	43.1 ± 4.2	62.0 ± 12.3	0	8.8 ± 3.1

TABLE 2

AVERAGE DISSOCIATION CONSTANTS (K_d) OF PROLACTIN BINDING TO ITS RECEPTORS IN MEMBRANE PREPARATION OF THE LIVER IN VIRGIN, PREGNANT AND LACTATING RATS

	× 10 M					
	Plasmalemma		Plasmalemma + Large Cell Organelles		Nuclear Membrane	
Animals	Unwashed	$MgCl_2$ Washed	Unwashed	$MgCl_2$ Washed	Unwashed	$MgCl_2$ Washed
Virgin, diestrus	1.94 ± 0.88	0.86 ± 0.17	1.24 ± 0.71	0.57 ± 0.29	2.11 ± 2.52	0.43 ± 0.34
Day 18 gestation	3.60 ± 2.27	1.00 ± 0.48	0.87 ± 0.24	0.42 ± 0.11	0.95 ± 0.29	0.42 ± 0.14
Day 1 lactation	1.30 ± 0.14	0.53 ± 0.10	0.88 ± 0.29	0.68 ± 0.26	1.30 ± 0.28	1.13 ± 0.17
Day 5 lactation	2.92 ± 0.85	2.41 ± 1.82	1.38 ± 0.56	0.68 ± 0.18	1.49 ± 0.01	0.65 ± 0.08
Day 12 lactation	1.29 ± 1.04	0.55 ± 0.13	1.13 ± 0.40	0.46 ± 0.24	0.06 ± 0.67	1.07 ± 1.06
Day 21 lactation	3.24(n=1)	0.95 ± 0.55	0.70 ± 0.24	0.54 ± 0.24	1.00(n=1)	0.41(n=1)
Day 30 lactation	1.22 ± 0.21	0.44 ± 0.09	0.66 ± 0.12	0.46 ± 0.24	0.94(n=1)	0.16 ± 0.01

the membrane and the dissociation constant (Kd) were calculated according to the principles of Scatchard [33]. For each determination, a regression coefficient (r) of the line was calculated from 5 points. The analysis was accepted only when $r \geq 0.90$ and $p < 0.01$.

Statistical Analyses

All numerical data were expressed as mean±SE. One-way analysis of variance and the Student-Neumann-Keuls test were applied to compare the differences among seven experimental groups. For analysis of total cell receptors, the nuclear membrane and the plasmalemma plus the large cell organelle fraction were added for each animal. To discuss the receptors in the large non-nuclear organelles, the mean value of plasma membranes was subtracted from the mean value of plasma plus large organelle membranes for each group.

RESULTS

Table 1 lists the average values of total binding capacity for three membrane preparations: plasmalemma, plasmalemma + large cell organelles and nuclear fractions. Values obtained from samples not washed by 5.0 M $MgCl_2$ represent the available binding sites, since those receptors already occupied by endogenous prolactin at the time of assay would not be displaced by the radiolabelled prolactin. Values obtained from samples which had been washed by 5.0 M $MgCl_2$ represent the total prolactin binding sites because $MgCl_2$ was able to dissociate previously bound hormone from the binding sites [13].

In virgin (diestrus) rats, the liver contained a considerable quantity of specific prolactin binding sites in all three membrane fractions. The majority of the sites was not occupied by endogenous prolactin, since there was no significant difference between the values obtained from the membrane fractions with or without washing by 5.0 M $MgCl_2$. There was a significant increase in specific prolactin binding in livers of rats at 18 days of gestation. This increase in receptor number occurred primarily in sites where endogenous prolactin had been previously bound. We know this since all membrane fractions showed a significant elevation in binding sites after washing with 5.0 M $MgCl_2$. The results for plasmalemma membranes closely resembled those reported by Van der Gugten *et al.* [37], Costlow *et al.* [6,7] and Carr and

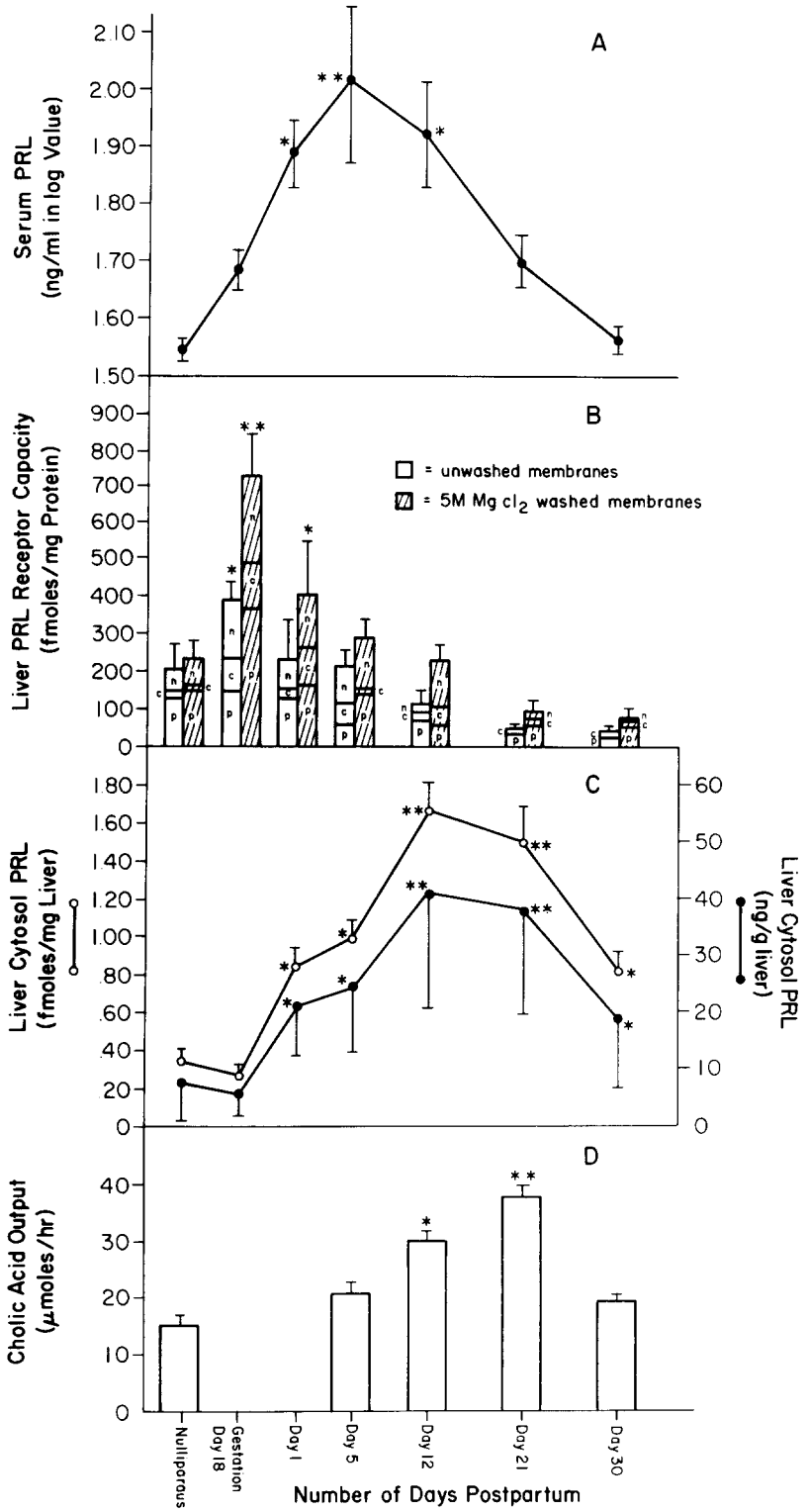

FIG. 1. Composite of serum concentrations of PRL (A), levels of prolactin binding to the liver membranes (B), liver cytosolic prolactin (C) and cholic acid output (D). Figure A and C from Lee, Lee and Moltz [19]. Figure D from Kilpatrick, Bolt and Moltz [17]. Figure B, n=nuclear membranes, c=other large organelle membranes, p=plasmalemma membranes. **Significantly greater than all other groups; *significantly greater than all other groups except **. Bars represent SEM. The data for serum PRL, liver PRL receptor capacity and liver cytosol PRL were collected from the same animals during a period of six months.

Jaffe [4] for virgin and pregnant rats. After parturition, prolactin binding in all membrane fractions declined persistently throughout the entire period of lactation. This decrease occurred not only in available binding sites but in total binding sites as well. During the latter part of lactation, prolactin binding sites in our liver specimens were reduced to a point below the sensitivity of our assay system.

Table 2 shows the average dissociation constant (K_d) of prolactin-receptor binding of the membrane fractions of the liver in rats of different physiologic states. Although the average dissociation constant for the $MgCl_2$-washed membranes is significantly less than that for the unwashed membranes ($p<0.01$) there were no significant differences among groups within each membrane fraction. This is important since it shows that we measured the same receptor under different physiological conditions.

DISCUSSION

The results of the present study indicate that prolactin binding to liver membrane fractions in the pregnant rat is significantly higher than in the nulliparous and lactating rat, respectively. Moreover, in the lactating rat a progressive decrease in prolactin binding occurs beginning on Day 1 postpartum. That this decrease was not an artifact of our experimental techniques can be seen in the fact that (a) the same procedures were applied to the livers of all animals, and (b) there was no change across groups in the affinity of the receptor sites since the dissociation constant (K_d) remained unchanged.

To relate our results to prolactin at the liver, we have prepared a composite figure that includes not only the data of the present experiment but data from several other studies carried out in our laboratory. Figure 1 shows serum concentrations, levels of prolactin binding to liver membranes, prolactin in liver cytosol and cholic acid output.

In the nulliparous rat, the prolactin receptor capacity of the liver was relatively low, concomitant with low levels of prolactin in serum and hepatic cytosol. Also evident was a relatively low level of cholic acid output. By Day 18 of pregnancy, however, prolactin binding in liver membranes was significantly heightened and, as Fig. 1 shows, a substantial number of the receptors were occupied by endogenous prolactin. However, this increase in receptor capacity was not accompanied by a significant increase in either serum prolactin or prolactin in hepatic cytosol. Thereafter, that is on Days 1, 5, 12, 21 and 30 postpartum, the prolactin receptor-capacity of the liver decreased progressively. And in fact on only one of these postpartum days, Day 1, did the liver show a receptor number significantly in excess of that seen in the nulliparous female. Yet on Day 5 postpartum, serum prolactin reached a peak level, and on Days 12 and 21 prolactin in hepatic cytosol was significantly elevated as was the output of cholic acid from the liver.

We had expected the prolactin binding capacity of the liver to rise commensurately with increases in circulating prolactin ("up-regulation"). Moreover, we thought that binding capacity would match the concentration of prolactin in hepatic cytosol, and in turn the heightened output of cholic acid from the liver. Instead, we found "down-regulation": as the concentration of prolactin in serum increased from pregnancy to early lactation the liver showed a decline in prolactin receptors. This decline continued, resulting in low receptor numbers when cytosolic prolactin and cholic acid reached peak levels.

Evident in Fig. 1 is the fact that by 21 days postpartum, serum prolactin was equivalent to that present at 18 days of pregnancy. Moreover on postpartum Day 21 prolactin receptor capacity was at its lowest level, while the concentration of prolactin in hepatic cytosol was markedly elevated. Three explanations might be advanced to account for this difference, although it is possible that none is true or that all three work in concert. First, the liver of the lactating rat undergoes changes which make it distinctively different from that of the pregnant rat [3,15]. Thus, unique biochemical constituents, which maximize liver responsiveness, may be available to the lactating female, but not to the pregnant female. Second, the rhythm of prolactin release differs markedly in the pregnant and lactating rat. During gestation, prolactin is released into the circulation in daily diurnal and nocturnal surges that occur at approximately equal intervals [10,38]. During lactation, multiple surges of prolactin occur in response to suckling [11]. These multiple surges may result in a down-regulation rather than in an up-regulation of prolactin receptors in liver. And finally, hormones other than prolactin distinguish pregnant and lactating rats. Perhaps one or more of these hormones influence the capacity of the liver to process circulating prolactin.

Djiane, Clausen and Kelly [9] demonstrated down-regulation of prolactin binding at the liver lasting only 12 hr, after injecting prolactin at a dose which raised blood levels of the hormone to 50–60 times the maximum ever measured in lactating rats. We, on the other hand, used undisturbed lactating animals and observed continuous down-regulation. Moreover, we observed down-regulation at a time of maximal liver responsiveness to prolactin. This heightened liver responsiveness to prolactin was identified by the presence of the pheromone in the feces of lactating rats between 14 and 27 days postpartum, and is connected with the elevated cytosolic accumulation of the hormone.

Although prolactin is bioactive when it first enters the cell, it is eventually degraded. Since we did not assess the potency of the hormone, it is possible that we were measuring immunoactivity but not bioactivity. However, the fact that large amounts of prolactin were found in the cell when receptor capacity was significantly depressed indicates that the liver was actively binding and transporting the hormone. Nonetheless, the question of what was being measured—active or inactive prolactin—should be clarified in future experiments.

A related question is that of RIA specificity: how likely is it that the crossreacting material in our assay system was not prolactin but an altogether different pituitary peptide? We are confident that it was not growth hormone, since, as Cowie *et al.* put it, "Mammalian prolactins do not show immunological cross-reactions with mammalian GHs" ([8] p. 24). Nor is it likely that any other pituitary peptide was crossreacting. Based on the data of NIAMDD attesting the high specificity of their RIA antiserum for prolactin (see Technical Report 117 of the Pituitary Hormone and Antisera Center, August 14, 1980) and on the RIA curves we found as well, there is little doubt that we were measuring prolactin.

Since we found peak levels of prolactin in liver cytosol only during a period of receptor down-regulation, this down-regulation is probably responsible for the intracellular accumulation of the hormone. Just how it promotes this accumulation remains unresolved. Also unresolved is how, after receptor down-regulation, heightened cytosolic prolactin elevates cholic acid, which leads to pheromonal emission. Experiments designed to answer these questions are currently being considered.

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