Effect of 9-Fluoroprednisolone Acetate and Transplanted Pituitaries on Milk Synthesis[1] (34866)

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Injections of either cortisone acetate (1) or cortisol acetate (2) cause significant increases in lactational performance during the first 18 days postpartum in rats. Cortisol-21-acetate injections (3) maintained mammary DNA and RNA content and retarded, but did not completely prevent, declines in litter weight gain during prolonged lactation (days 16 to 32). The present experiments were designed to determine if variable doses of a synthetic glucocorticoid (9-fluoroprednisolone acetate) would fully maintain mammary cell numbers (DNA) and secretory activity (RNA, RNA/DNA, and litter wt gain) during prolonged lactation. Another aspect of these experiments was to determine if livers from 9-fluoroprednisolone acetate-treated rats metabolize cortisol to a greater degree than livers from saline-injected control rats.

Contrary to the effects of cortisol-21-acetate on lactation, we (3) observed that prolactin did not exert any marked galactopoietic influence on litter weight gain during prolonged lactation. Since the prolactin was of ovine origin and administered only twice daily, another objective of the present research was to determine if the chronic secretion of rat prolactin from pituitaries isotransplanted under the kidney capsule (4) of lactating rats would prevent declines in milk synthesis during prolonged lactation.

Materials and Methods. On day 3 of lactation, thoracic teats of all rats were ligated, litter size was adjusted to 6 pups and mother rats were weighed. At days 16 and 24 of lactation, litters were replaced with 8-day-old foster litters to maintain an intense suckling stimulus. Litter weights were recorded daily. Cumulative litter weight gains were calculated from day 16 to 24 and from day 24 to 32 of lactation. All mother rats were decapitated on day 32 of lactation and the six abdominal-inguinal mammary glands were removed, weighed, trimmed, and stored in 0.25 *M* sucrose at —20° until analyzed for nucleic acid content (5). Body, anterior and posterior pituitary, adrenal, ovary, and uterine weights were recorded at autopsy.

Expt. 1. Injections of 9-fluoroprednisolone acetate (Predef)[3] were given twice daily from day 16 to 32 (autopsy) of lactation at doses of either 0 (0.85% NaCl), 10, 50, or 100 μg/day. The number of rats in each group was 10, 9, 10, and 8, respectively.

Expt. 2. Since Predef injections in Expt. 1 only partially retarded declines in litter weight gain from the first (16 to 24 days) to second 8-day period (24 to 32 days) of lactation, a second experiment was designed to determine if these declines could be minimized by delaying the onset of Predef injections or by doubling the dose of Predef from the first to second 8-day period. Ten control lactating rats received 0.85% NaCl throughout the 16–32 day treatment period. A second group of 13 rats received NaCl injections between days 16 and 23 and 50 μg of Predef daily between days 24 and 32 of lactation. A third group of 10 rats was injected daily with 50 μg of Predef from day 16 to 23 and 100

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μg of Predef from day 24 to 32 of lactation.

To determine if livers from Predef-injected rats metabolize more cortisol than livers of control rats a 0.5-g section of each of the livers of rats from the first and third groups was frozen at —20° for subsequent protein measurements (6). The remaining part of the livers was homogenized, for two 3-sec intervals in 4 vol of 0.1 *M* phosphate buffer containing 20% glycerol, pH 7.4 (phosphate–glycerol buffer).

The homogenate was centrifuged at 9000*g* for 20 min at 5°. The 9000*g* supernatant fluid was then centrifuged at 105,000*g* for 60 min at 5° to produce a microsome pellet. This supernatant fluid was frozen at —20°. Microsome pellets were rinsed once with phosphate–glycerol buffer, homogenized with a volume of phosphate–glycerol buffer equal to 0.5 ml times the number of microsome pellets, and then frozen at —20°.

On the day of assay, 2-ml aliquots from each microsome and 105,000*g* supernatant preparation were pooled within treatments. The results of a preliminary experiment indicated that an incubation with 1 ml each of microsome and 105,000*g* supernatant preparations increased the amount of cortisol metabolized by 144% in comparison with an incubation with microsomes alone. Consequently, all subsequent incubations were performed with 1 ml of the pooled microsome fraction plus 1 ml of the pooled 105,000*g* supernaant fraction.

Pooled liver microsomes plus 105,000*g* supernatant fluids from saline- (group 1) and Predef-treated (group 3) rats were each incubated with three levels of a mixture of cortisol[4] and cortisol-^{14}C.[5] The three levels of substrate mixture used were: 1 μ*M* (1.0912 μCi/mg), 2 μ*M* (0.548 μCi/mg) and 5 μ*M* (0.219 μCi/mg). Isocitric dehydrogenase, isocitric acid, and NADP (Sigma) were used as a NADPH generating system during the incubation.

Prior to assay, 30 ml of 0.1 *M* phosphate buffer without glycerol, pH 7.4 (phosphate buffer), was gassed for 5 min with 95% O_2–5% CO_2. Treatment tubes contained 2.5 ml of phosphate buffer, 0.1 ml of $MgCl_2$ (15 μ*M*), 0.1 ml of NADP (1.00 μ*M*), 0.1 ml of isocitric acid (10 μ*M*, 0.2 ml of isocitric dehydrogenase (3 units), 1, 2, or 5 μ*M* cortisol-^{14}C, 1.0 ml of pooled microsomes and 1.0 ml of pooled 105,000*g* supernatant fluid. Blank tubes contained: 4.5 ml of phosphate buffer, 0.1 ml of $MgCl_2$ (15 μ*M*), 0.1 ml of NADP (1.00 μ*M*), 0.1 ml of isocitric acid (10 μ*M*), 0.2 ml of isocitric dehydrogenase (3 units) and 1 or 5 μ*M* cortisol-^{14}C. All tubes were gassed for 30 sec with 95% O_2–5% CO_2 and incubated for 1 hr at 37° in a Dubnoff shaker. Reactions were terminated with 1 ml of 1 *N* HCl. Incubation media and contents were directly shaken with 30 ml of methylene chloride for 30 min, and centrifuged at 1650*g* for 15 min.

Twenty-four ml of the methylene chloride extract were taken to dryness under nitrogen at 45°. The dried residue was redissolved in 1 ml of ethanol. Fifty μl of the ethanol extract was spotted on silica gel thin-layer chromatography plates (chromatogram sheet 6060).[6] Chromatograms were developed in chloroform:methanol:water (90:10:1) for 45 min. Cortisol was visualized with ultraviolet light. Excluding the cortisol spot, 1-cm strips were cut from the origin to solvent front. Each strip, including the cortisol spot, was placed directly in scintillation vials and radioactivity was quantified in a Model 6725 Nuclear Chicago liquid scintillation spectrometer.

Distribution of dpm from origin to solvent front was used as a quantitative measurement of the amount of cortisol metabolized as well as the amount of polar or nonpolar metabolites formed. The area from the origin to the cortisol spot was designated as polar; from the cortisol spot to the solvent front was designated the nonpolar metabolite area.

Expt. 3. On day 3 of lactation, 5 anterior pituitaries from mature female rats were transplanted under the left kidney capsule of each of 14 lactating rats. Kidneys of 11 sham-operated controls were manipulated

[4] Sigma Chemical Company, St. Louis, Missouri.

[5] Nuclear-Chicago Corporation, Des Plaines, Illinois.

[6] Distillation Products Industries, Rochester, New York.

TABLE I. Nucleic Acid Content of Mammary Glands and Litter Weight Gain of Intensely[a] Nursed Rats Injected with 9-Fluoroprednisolone Acetate (Predef) Between Days 16 and 32.

	Predef (μg/day)[b]			
	Saline	10	50	100
Litter wt gain[c] (g)				
16–24 days	71.4 ± 5.9	87.7 ± 5.0	105.8 ± 5.6	115.0 ± 5.4
24–32 days	32.2 ± 4.9	54.6 ± 5.8	71.2 ± 8.6	73.2 ± 4.2
Body wt gain (g)				
18–32 days	6.7 ± 3.0	—1.4 ± 6.8	—13.0 ± 3.8	—35.0 ± 5.4
Total DNA (mg)	24.1 ± 1.3	26.0 ± 2.4	39.9 ± 2.3	28.8 ± 1.7
Total RNA (mg)	134.6 ± 7.7	169.7 ±15.8	202.5 ±13.4	197.1 ±14.7
RNA/DNA	5.6 ± 0.2	6.6 ± 0.3	5.1 ± 0.3	7.0 ± 0.7

[a] Litters 16 days old replaced with 8-day-old foster litters.
[b] Mean and SE of mean.
[c] Cumulative litter weight gains were recorded between days 8 and 16 of age for all litters.

surgically in a manner similar to that of rats receiving pituitary transplants. Vaginal smears were recorded daily. Both groups of rats were killed on day 32 of lactation and the usual mammary and organ data were collected.

Results. Expt. 1. The 16–24-day litter weight gains for rats given 50 or 100 μg of Predef daily were significantly greater ($p < 0.01$) than litter weight gains of rats injected with 10 μg of Predef or saline, respectively (Table I). The 10-μg dose caused a greater ($p < 0.05$) litter weight gain response than saline injections. During the second 8-day period (24 to 32 days of lactation) 50 and 100 μg of Predef increased litter weight gain approximately 124% above values for saline-injected rats. In addition, 10 μg caused a significant increase ($p < 0.05$) in litter weight gain over that of saline-injected control rats. However, there was a decline in cumulative litter weight gain from the first to the second 8-day period in all treatments.

Fifty μg of Predef maintained mammary DNA of the lactating rats at a level higher ($p < 0.01$) than that in rats receiving either 10 μg of Predef or saline. However, 100 μg of Predef failed to maintain mammary DNA at a level comparable with that observed in the 50-μg treated rats.

Changes in mammary RNA content of the lactating mother rats for the various treat-

TABLE II. Nucleic Acid Content of Mammary Glands and Litter Weight Gain of Intensely[a] Nursed Rats Injected with 9-Fluoroprednisolone Acetate (Predef) Between Days 16 and 32.

Days of lactation:	16–23	24–32[b]	16–23	24–32[b]	16–23	24–32[b]
Predef (μg):	0	0	0	50	50	100
Litter wt gain[c] (g)						
16–24 days	81.1 ± 4.8		89.0 ± 5.4		92.5 ± 4.6	
24–32 days	42.8 ± 3.5		83.0 ± 5.1		83.7 ± 7.2	
Body wt gain (g)						
18–32 days	9.1 ± 1.5		—18.3 ± 2.8		—19.7 ± 4.3	
Total DNA (mg)	28.3 ± 1.2		32.8 ± 1.8		31.2 ± 1.5	
Total RNA (mg)	140.7 ± 6.6		206.2 ± 9.0		180.4 ±13.3	
RNA/DNA	5.1 ± 0.4		6.4 ± 0.2		5.8 ± 0.2	

[a] Litters 16 days old replaced with 8-day-old foster litters.
[b] Mean and SE of mean.
[c] Cumulative litter weight gains were recorded between days 8 and 16 of age for all litters.

ments paralleled the 24–32-day litter weight gain responses. Thus, rats treated with either 50 or 100 μg of Predef contained more ($p < 0.01$) mammary gland RNA than rats receiving 10 μg of Predef or saline. Ten μg of Predef increased ($p < 0.08$) RNA content above the value for the saline control group.

Increasing the dose of Predef caused a linear decline ($p < 0.01$) in body weight of the mother rats from day 18 to 32 of lactation. Adrenal weights of rats injected with 100 μg of Predef were less ($p < 0.05$) than adrenal weights of rats receiving 0, 10, or 50 μg of Predef. No significant differences ($p > 0.05$) were detected for anterior pituitary, ovarian, or uterine weights.

Expt. 2. The 24- to 32-day litter weight gain response of the second and third treatment groups (Predef) was 95% greater than that of saline-injected rats (Table II). Injections of 50 μg of Predef daily from 24 to 32 days of lactation (group 2) resulted in a litter weight gain response comparable to that in group 3. Rats receiving Predef had more mammary DNA ($p < 0.05$) and more RNA ($p < 0.01$) than saline-injected controls. Rats in group 3, which received the highest dose of Predef for the longest time, had adrenal weights which were significantly less ($p < 0.01$) than those of either groups 1 or 2. Anterior and posterior pituitary and ovarian weights did not differ significantly ($p > 0.05$) among the three groups.

Liver preparations of Predef-treated rats (group 3) incubated at the 1-, 2-, and 5-μ*M* cortisol substrate levels consistently metabolized a greater percentage of cortisol to polar metabolites and a smaller percentage to nonpolar metabolites in comparison with liver preparations of saline-treated rats (Table III).

Total liver weights, liver protein content, and protein per gram of liver did not differ ($p > 0.05$) between Predef- and saline-injected rats (Table IV). Liver weights per 100 g of body weight were significantly larger ($p < 0.025$) in Predef-injected rats than in saline-injected rats. Pooled microsomal protein contents of saline-injected rats were 144% greater than the pooled microsomal

TABLE III. Amount of Cortisol Metabolized per Hour from Liver Preparations of Intensely[a] Nursed Rats Injected with Saline and 9-Fluoroprednisolone Acetate (Predef) Between Days 16 and 32.

Pretreatment from 16–32 days of lactation	Cortisol substrate added (μ*M*)	Total cortisol metabolites produced in methylene chloride phase		Nonpolar cortisol metabolites produced in methylene chloride phase		Polar cortisol metabolites produced in methylene chloride phase	
		(μ*M*/hr)	(mμ*M*/hr/mg of protein)	(μ*M*/hr)	(mμ*M*/hr/mg of protein)	(μ*M*/hr)	(mμ*M*/hr/mg of protein)
Saline	1	0.448	16.6	0.149 (33%)[b]	5.5 (34%)	0.298 (67%)	11.0 (66%)
	2	0.555	22.1	0.254 (46%)	11.0 (50%)	0.301 (54%)	11.0 (50%)
	5	0.591	22.1	0.348 (59%)	13.8 (62%)	0.243 (41%)	8.3 (38%)
Predef	1	0.351	24.9	0.028 (8%)	2.8 (11%)	0.323 (92%)	22.1 (89%)
	2	0.671	47.0	0.108 (16%)	8.3 (18%)	0.564 (84%)	38.7 (82%)
	5	0.470	33.1	0.086 (18%)	5.5 (17%)	0.384 (82%)	27.6 (83%)

[a] Litters 16 days old replaced with 8-day-old foster litters.
[b] Values in parentheses are percentage of total metabolites.

TABLE IV. Liver Weights and Protein Measurements of Liver Microsomes and 105,000*g* Supernatant Fluid of Intensely[a] Nursed Rats Injected with Saline and 9-Fluoroprednisolone Acetate (Predef) Between Days 16 and 32.

	Predef	Saline
Liver wt (g)	11.0 ± 0.2[b]	11.4 ± 0.3[b]
Liver wt (g/100 g of body wt)	4.9 ± 0.1	4.6 ± 0.1
Total liver protein (mg)	1896 ±139	1934 ±55
Liver protein concentration (mg/g)	173 ± 13	183 ± 2
Pooled microsomal protein (mg/ml)	6.1	14.9
Pooled 105,000*g* supernatant (mg/ml)	8.0	10.6

[a] Litters 16 days old replaced with 8-day-old foster litters.

[b] Mean and SE of mean.

protein content of Predef-treated rats.

Expt. 3. Vaginal smears of rats containing five anterior pituitary transplants and of sham-operated controls indicated that both groups were in an anestrous state throughout lactation. The 16–24- and 24–32-day litter weight gain responses for rats with pituitary transplants were not significantly different from sham-operated rats ($p > 0.05$) at either the 16–24 or the 24–32-day periods (Table V). On the other hand, anterior pituitary transplants significantly increased ($p < 0.05$) mammary gland DNA, RNA, and RNA/DNA ratios over sham-operated control rats.

Anterior pituitary and ovarian weights of rats with anterior pituitary transplants were significantly less ($p < 0.05$) than those of sham-operated controls. There were no significant differences in body weight gains, adrenal or uterine weights of the mother rats associated with the transplants.

Discussion. In Expt. 2, the 50-μg dose of Predef maintained cell numbers and total metabolic activity of the mammary gland. However, synthetic activity per cell was reduced. The 100-μg dose of Predef in comparison with the 50-μg dose appeared to overstimulate the mammary gland as reflected by a loss in DNA and a higher synthetic activity of the remaining cells.

Varying the dose of Predef during the treatment periods, by either a delay in onset of injections to day 24 of lactation or an increase in dose of Predef during the treatment period minimized declines in litter weight gain (Expt. 2). These results *in vivo* indicated that daily hormone injections during the first 8-day period increased the corticoid dose necessary to maintain lactation during the second 8-day period. Recent studies (7) have demonstrated the ability of phenobarbital and other liver microsomal enzyme inducers to inhibit the actions of both exogenous and endogenous steroids *in vivo.* The results of the present study indicate that injections of a synthetic glucocorticoid into lactating rats stimulates the liver to metabolize cortisol to polar metabolites when tested *in vitro.* These polar metabolites are probably biologically less active and more water soluble and thus, more readily excreted. Consequently, an increased rate of corticoid metabolism may partially account for the in-

TABLE V. Nucleic Acid Content of Mammary Glands, Litter Weight Gain and Organ Weights of Intensely[a] Nursed Rats Bearing Isotransplanted Pituitaries Between Days 3 and 32.

	Pituitary transplant[b]	Sham operation[b]
Litter wt gain[c] (g)		
16–24 days	79.4 ± 5.9	83.3 ± 4.3
24–32 days	40.4 ± 5.3	24.5 ± 6.8
Body wt gain (g)		
18–32 days	6.2 ± 3.0	−1.0 ± 2.6
Total DNA (mg)	29.2 ± 1.1	24.0 ± 1.7
Total RNA (mg)	138.2 ±10.0	98.2 ±10.7
RNA/DNA	4.7 ± 0.2	4.0 ± 0.2
Anterior pituitary (mg)	8.5 ± 0.3	9.6 ± 0.3
Adrenal (mg)	51.8 ± 1.7	54.0 ± 2.6
Ovary (mg)	64.9 ± 3.1	78.3 ± 5.2
Uterus (mg)	266 ±17	316 ±20

[a] Litters 16 days old replaced with 8-day-old foster litters.

[b] Mean and SE of mean.

[c] Cumulative litter weight gains were recorded between days 8 and 16 of age for all litters.

creased Predef dose necessary to maintain lactation in Expt. 2.

Anterior pituitaries transplanted to the kidney capsule synthesize and release copious amounts of prolactin (4). The decrease in pituitary weight and increase in mammary DNA and RNA content of rats bearing transplanted pituitaries indicated that the transplanted pituitaries were functional during lactation. These results are in agreement with those from virgin rats (8). However, secretion of rat prolactin from the transplants in the present experiment failed to elicit a galactopoietic effect during advanced lactaion. Cowie *et al.* (9) also reported that pituitary grafts under the kidney capsule had no effect on milk yield during early lactation. Although prolactin is needed for lactation, it is probably not normally rate limiting to milk synthesis in the rat because pituitary prolactin content does not decrease during extended lactation (10), and neither ovine prolactin injections (3) nor rat prolactin from isotransplanted pituitaries increases milk yield.

Summary. A daily dose of 50 μg of 9-fluoroprednisolone acetate (Predef), from days 16 to 32 of lactation, retarded the normal decline in litter weight gain and maintained rat mammary gland DNA and RNA content. A delay in the onset of injections until day 24, or injections of 50 μg of Predef daily from days 16 to 23 and 100 μg of Predef daily from 24 to 32 days of lactation maintained litter weight gain and increased mammary gland nucleic acid content. Predef injections for 16 days increased the ability of *in vitro* liver preparations to metabolize cortisol into polar metabolites while formation of nonpolar metabolites was reduced. Chronic secretion of rat prolactin from five isotransplanted pituitaries under the kidney capsule did not increase litter weight gain. However, marginal increases in mammary gland DNA and RNA content and RNA/DNA ratio were detected.

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