A Rapid Assay for the Lactogenic Activity of Rat Chorionic Mammotropin* (32888)

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The luteotropic substance present in the rat placenta at midpregnancy and first described by Astwood and Greep (1) was demonstrated to be also mammotropic and to have its highest potency in trophoblastic tissue on day 12 of pregnancy by Ray, *et al.* (2). Matthies (3) studied rat chorionic mammotropin (RCM) and found it to be a protein present also in maternal peripheral blood on days 11, 12, and 13 of pregnancy with a peak activity on day 12 (day 1 was the day of sperm detection in the vaginal smear). In that investigation, luteotropic and mammogenic activities were demonstrated by the deciduoma reaction and lobulo-alveolar mammary growth, respectively, in the hypophysectomized rat. Lactogenic activity was demonstrated by mammary secretory activity, detectable as alveolar distention, when maternal serum or trophoblastic extracts containing prednisolone acetate were injected into hypophysectomized, ovariectomized, adrenalectomized rats. The entire trophoblastic component of 1 day 12 conceptus was found to contain approximately the same amount of activity as 0.1 ml of maternal serum from the same donor rat. Sera from days 11 and 13 were found to contain approximately one third of that activity.

Because RCM does not produce a strong pigeon crop-sac response, another rapid and convenient bioassay method was sought for in the course of purification procedures. Lyons, *et al.* (4) had shown that ovine pituitary lactogenic hormone plus hydrocortisone acetate would induce mammary secretion in hypophysectomized, ovariectomized, adrenalectomized (triply operated) female rats and also in triply operated immature males. This suggested that the male rat might possess advantages in tests for lactogenic activity if the well-known inhibitory action of certain levels of the combined ovarian steroids on milk secretion, recently reviewed by Barnawell (5), were to be avoided. It was found that the pituitary need not be removed when a single mammary gland is treated locally with the test solution, and the contralateral, untreated mammary tissue is used as a control.

Materials and Methods. Male Long-Evans rats 30 days old were used as assay animals and all test solutions contained 20 μg prednisolone acetate, per dose, in microcrystalline suspension. Because this assay depends upon a comparison between the treated gland and the contralateral, untreated control gland, the injection must be made with care so that the solution is placed in the immediate area of the test gland and not in the surrounding adipose tissue. The nipple—a convenient guide to mammary tissue in the female rat—is absent in the male, but the fourth, right mammary gland is located just medial to the knee when the thigh is flexed on the abdomen. A few trial injections with a dye such as trypan blue provides the experience necessary for accurate placement of the test solution. A group of lymph nodes marks the lateral border of the fourth gland and is used to locate the gland macroscopically upon dissection. The injected solution, placed in the subcutaneous connective tissue, produces an elevation of the skin seen best by prior removal of the pelage. A 26-gauge, 0.5-inch hypodermic needle introduced subcutaneously near the midline of the abdomen is freely movable in the connective tissue and may be accurately directed to the injection site.

The prednisolone was added directly to the test solution and resuspended by agitation before being drawn up for injection. The dose volume was adjusted to 0.2 ml with 0.85% saline solution in order to cover the area of the fourth gland. Volumes much in excess of this were subject to partial loss by reflux unless a

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longer needle was used in making a more extensive subcutaneous approach to the injection site. The contralateral, control gland may be injected with prednisolone only, but this was found to be unnecessary because, in the absence of an exogenous lactogen, the mammary gland of these animals did not respond to prednisolone in doses of 50 μg.

In most cases injections were made once daily for 4 days, although the response to 3 days, 2 days, and 1 day of treatment was also tested. At autopsy on the fifth day, the right and left mammary glands were dissected from the skin flaps as whole mounts, fixed in buffered 10% formalin on filter paper, clipped together, stained with alum carmine, and cleared in methyl salicylate as described by Lyons and Johnson (6). Whole mounts so prepared can be processed later for histological study.

Neither castration nor adrenalectomy of the assay animal impaired the response of the mammary gland to extracts containing RCM and prednisolone, but hypophysectomy significantly reduced alveolar distention.

Results. The assay procedure described above demonstrates the lactogenic property of RCM through its synergy with prednisolone. Extracts containing these two hormones induce mammary secretion as evidenced by the distention of the alveoli of the treated gland and the absence of distention in the con-

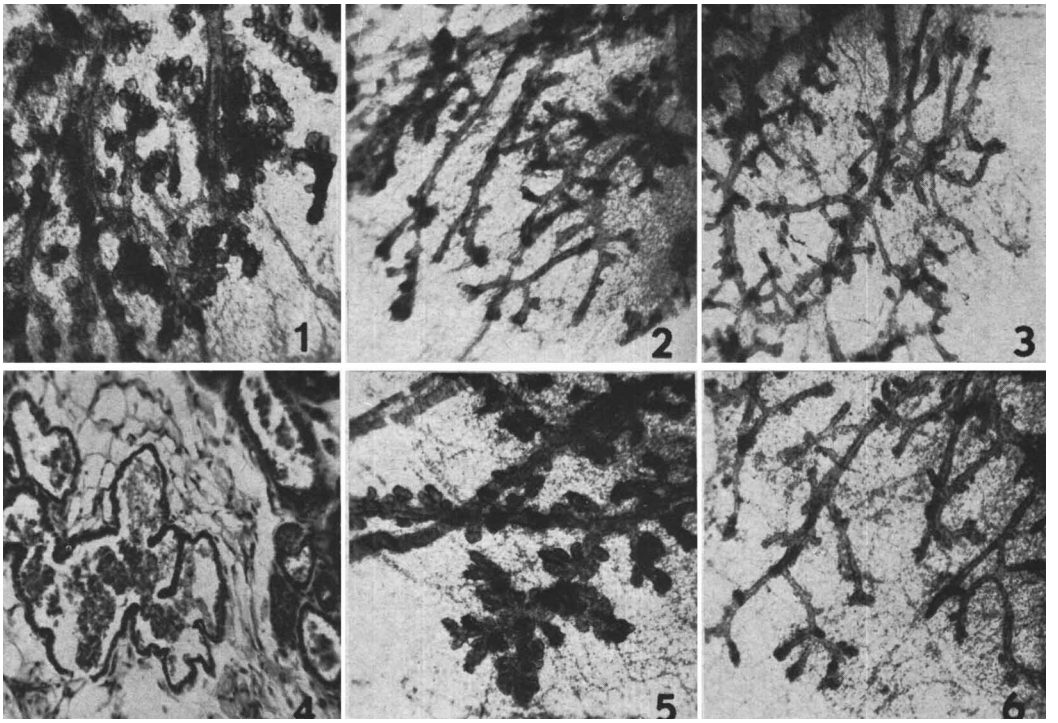

FIG. 1. Right, fourth mammary gland of immature male rat illustrating distended alveoli resulting from four local injections of 0.1 ml of day 12 pregnancy serum plus 20 μg of prednisolone. ×20.

FIG. 2. Contralateral, untreated control gland from same animal as Fig. 1. ×20.

FIG. 3. Right, fourth mammary gland treated for 4 days with 20 μg prednisolone in saline. ×20.

FIG. 4. Histological section of milk-distended alveoli of gland treated for 3 days with 0.5 placental equivalents of day 12 trophoblast plus 20 μg of prednisolone. ×100.

FIG. 5. Right, fourth mammary gland treated for 4 days with 0.005 ml of day 12 serum plus 20 μg of prednisolone. Distended alveoli were not present in the contralateral gland. ×20.

FIG. 6. Right, fourth mammary gland treated for 4 days with 0.5 ml of day 5 serum plus 20 μg of prednisolone. No alveolar distention. ×20.

tralateral control gland of the same animal. Figure 1 illustrates the response of the fourth right mammary gland to the local injection for 4 days of 0.1 ml of aortic blood serum from the 12-day pregnant rat plus 20 μg of prednisolone. This degree of alveolar distention was designated "plus 3" on an arbitrary scale of 0–4. Figure 2 shows the contralateral, untreated control gland in which distended alveoli are absent. Figure 3 shows a gland in which 20 μg of prednisolone per day for 4 days did not produce mammary alveolar distention in the absence of the exogenous synergistic lactogen. Glands treated with extracts of the fetal portion of the day 12 rat placenta plus prednisolone (Fig. 4) responded in a manner similar to those treated with the active serum.

By use of this assay it has been possible to consistently detect RCM in 0.005 ml of day 12 pregnancy serum in doses diluted to 0.2 ml with saline and containing 20 μg of prednisolone (Table I). Figure 5 illustrates a typical response to 4 days of local treatment with this dosage. One-half of this dosage did not consistently produce a positive response. Evidence that the assay does not detect circulating pituitary mammotropin is provided by the absence of alveolar distention in the mammary gland treated with prednisolone plus 0.5 ml of serum taken from rats on the fifth day of pregnancy (Fig. 6). Sera from rats 15-days

TABLE I. Alveolar Distention in Glands of Young Male Rats Treated with Prednisolone and Solutions Containing RCM.

Material injected (units)	Dose per injection	No. of days	No. of inj.	No. of rats	No. positive	Mean alveolar distention[a]
Serum: day 12 preg. (ml)	0.2	4	4	10	10	2.8
	0.10	4	4	9	9	2.6
	0.05	4	4	10	10	2.1
	0.025	4	4	11	10	1.6
	0.0125	4	4	7	7	1.4
	0.005	4	4	10	10	1.2
	0.0025	4	4	10	6	<1.0
	0.10	3	3	11	11	2.2
	0.10	2	2	5	5	2.3
	0.10	2	8	5	5	3.4
	0.10	1	1	5	1	<1.0
	0.10	1	4	5	3	<1.0
Plac. homog.[b] day 12 (placental eq.)	0.50	4	4	5	5	2.8
	0.25	4	4	6	6	2.0
	0.10	4	4	5	5	1.9
	0.05	4	4	6	6	1.6
	0.50	3	3	6	6	2.2
Ant. pit. homog.[c] (pituitary eq.)	0.25	4	4	4	4	2.1
	0.10	4	4	3	3	<1.0
Serum: day 5 preg. (ml)	0.5	4	4	6	0	Neg.
Serum: day 15 preg.	0.5	4	4	4	0	Neg.
Serum: nulliparous	0.5	4	4	4	0	Neg.
Pred. in saline (μg)	20	4	4	6	0	Neg.
	50	4	4	5	0	Neg.
	100	4	4	10	4	<1.0
	20	10	10	3	2	1.0

[a] Mean alveolar distention of treated gland based on arbitrary scale of 0–4.

[b] Supernatant from centrifuged homogenate of trophoblastic tissue, in placental equivalents.

[c] Supernatant from centrifuged homogenate of maternal anterior pituitary in pituitary equivalents.

pregnant and from nulliparous rats also failed to evoke a response at 0.5 ml per day. The animals do respond, however, to crude homogenates of anterior pituitary tissue (Table I).

Table I demonstrates that by the use of an arbitrary index of the degree of alveolar distention, semiquantitative data may be derived for comparison of potency of extracts containing RCM. Furthermore, it is evident from the table that this response is dependent not only upon dosage but also upon time. A good response was elicited by 0.1 ml of day 12 pregnancy serum in 2 days with two injections but not in 1 day, even though the animals were injected four times. The 2-day response was better when four 0.1 ml injections were administered daily than when the animals were injected only once per day. Prednisolone in dosages of 50 μg locally injected daily for 4 days did not induce mammary alveolar distention. Four of ten animals, however, responded slightly to 100 μg of prednisolone administered for 4 days. Prednisolone in dosages of 20 μg/day was capable of inducing mammary alveolar distention after prolonged (10 days) treatment.

Discussion. In addition to rapidity and simplicity, the assay of the lactogenic property of RCM described here has approximately 10× the sensitivity of the test for mammogenic activity and 20× the sensitivity of the test for luteotropic activity in the hypophysectomized female rat reported earlier (3). The failure of 0.5 ml of serum, taken from pregnant rats prior to implantation to produce the lactation response demonstrates that the test will not respond to serum free of RCM at 100× the volume of day 12 serum in which the hormone is detectable. Furthermore, circulating levels of pituitary lactogens in such sera do not produce a positive response nor are they detectable in 0.5 of serum from nulliparous rats. They are assayable in the form of crude anterior lobe homogenates, however, and may synergize with RCM as suggested by the decrease in the sensitivity of the assay which has been experienced with hypophysectomized animals. Utilizing this test, the presence of RCM at levels lower than those found at day 12 has been detected in the placenta and maternal blood of rats pregnant 9 through 14 days, confirming the observations of Ray *et al.* (2). The sensitivity of the test has enabled detection of RCM in dilute fractions from purification procedures without the necessity of volume reduction. The importance of this observation lies not only in its convenience but also in the fact that the freezing of extracts containing RCM severely depletes its biological activity, thus precluding the use of a number of volume reduction techniques.

Although the assay animal described above serves adequately as a presumptive test for the lactogenic activity of RCM, confirmation of the lactational response should be made in the triply operated rat.

Summary. A sensitive bioassay for the lactogenic activity of rat chorionic mammotropin, a secretion of the midpregnancy rat placenta, utilizes the synergism of this hormone with prednisolone acetate to induce mammary secretion in the intact, immature, male rat. Activity in locally injected solutions is detected by the appearance of distended alveoli in stained whole mounts of the treated mammary gland. The contralateral, untreated gland serves as a control. Reliable responses to extracts containing RCM have been produced in 2–4 days, depending upon dosage and number of injections.

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