Radioimmunoassay for Rat Prolactin* (33657)

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The most commonly used method for measuring prolactin in biological samples has been the pigeon crop bioassay of Lyons (1). It is possible with this method to estimate the prolactin content of pituitary preparations and of other tissues containing relatively high concentrations of this hormone (a minimum of about 0.03 IU or 2 μg of NIH-P-B1, 13 IU/mg); however, it was not possible to assay the prolactin present in serum from normal animals. Recently, double antibody radioimmunoassay techniques have been developed for ovine (2) and bovine prolactin (3) and a radioimmunoassay for rat prolactin (4) which uses a chromatoelectrophoresis step to separate free prolactin-^{125}I from that bound to the antibody has also been reported. These techniques are sufficiently sensitive to quantitate serum levels of prolactin. This communication describes the development of a double antibody radioimmunoassay for rat prolactin capable of measuring the amount of prolactin in 100 μl of serum from immature female rats or in 25 μl of serum from normal females during diestrus.

Methods and Materials. Purified rat prolactin (HIV-8-C) for radioiodination and for immunization was prepared from whole, frozen rat pituitary glands by Ellis *et al.* (5). This preparation is designated B329 in the remainder of the present study. Other preparations with differing concentrations of rat prolactin were obtained by physiologic manipulation of three groups of mature, female Sprague-Dawley rats. Rats in the first group (B350) were ovariectomized and injected subcutaneously with 10 μg of estradiol benzoate in corn oil daily for 10 days. Rats in the second group (B349) were ovariectomized and injected with corn oil for 10 days while the third group was composed of untreated intact females during diestrus (B351). At the end of 2 weeks the rats were bled terminally via the abdominal vena cava, and the blood from each group was pooled and serum was obtained by centrifugation. Pituitary glands from each group were pooled and homogenized in 0.01 *M* phosphate buffer in 0.14 *M* NaCl (PBS). Pituitary glands and sera were also obtained from groups of immature female (B400) and mature male (B401) rats. Purified preparations of rat luteinizing hormone (B301, 0.8 times as potent as NIH-LH-Sl) (6), follicle stimulating hormone (B341), 70 times as potent as NIH-FSH-Sl (6), and growth hormone (HVII-38-C, 3.9 USP units/mg; designated B330) were also used as test preparations for this radioimmunoassay.

The prolactin content of all pituitary preparations was bioassayed by the local intradermal pigeon crop bioassay of Lyons (1) as modified by Reece and Turner (7). Each sample was injected into one side of the pigeon crop and bovine prolactin (NIH-P-B1) or rat prolactin (B329) was injected into the other side. All preparations were bioassayed 3 times against standard bovine prolactin and rat prolactin. Pituitary homogenates were assayed at a concentration of 10 mg/ml. Each of 4 daily injections was made in 0.1 ml so that the total pituitary tissue given was 4 mg/pigeon.

Antiserum against rat prolactin was prepared in 6 adult female rabbits by 3 subcutaneous injections of 0.1–0.4 mg of purified rat prolactin (B329) emulsified in complete Freund's adjuvant and injected at intervals of 3 weeks. Antirabbit gamma globulin (RGG) was prepared in sheep as previously described (8).

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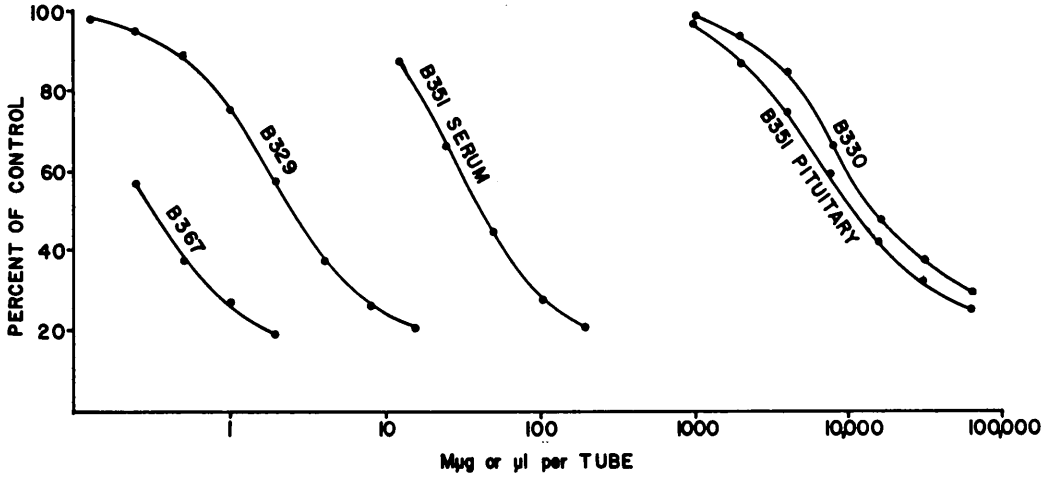

FIG. 1. Dose–response curves for prolactin in rat pituitary preparations and serum.

The methods for radioiodination and radioimmunoassay were the same as those described previously for rat luteinizing hormone (9) with the exception that 20 μg of Chloramine-T were used for radioiodination of rat prolactin and PBS-1% lyophilized egg white (EW) (Sigma Chemical Co.) was used rather than PBS-1% bovine serum albumin. In brief, the assay involved the addition of the sample or standard in 500 μl of PBS-1% EW. The antibody was diluted 1:400 in 0.05 *M* EDTA–PBS and then further diluted to 1:4000 in 1:400 normal rabbit serum in 0.05 *M* EDTA–PBS. The assay tubes received 200 μl of the diluted antibody and were stored at 4° for 24 hr. Rat prolactin-^{131}I (100 μl), diluted to give 65,000 cpm in a Nuclear Chicago , Automatic Gamma counting system with a 3-in. crystal on the day after radioiodination, was added to each tube and they were again stored at 4°. After an additional 24 hr, 200 μl of sheep anti-RGG, at a dilution which maximally precipitated the RGG present, were added to each tube. To ensure that complete precipitation was obtained in all tubes, incubation was allowed to continue for an additional 72 hr. Three ml of PBS were then added, the tubes were centrifuged and the supernatant was decanted and discarded.

Results and Discussion. A single band of radioactivity was noted following radioiodination and electrophoresis in polyacrylamide gel of the rat prolactin (B329). Under conditions of antibody excess (1:400 dilution) 91% of the rat prolactin-^{131}I was bound to the antibody, whereas at the working concentration (1:4000 dilution) approximately 60% of the prolactin-^{131}I was antibody bound.

The quantity of rat prolactin-^{131}I bound to the antibody in the absence of competing unlabeled hormone (i.e., PBS-EW control tubes) was considered to represent 100%. Dose–response curves depicting the decrease in the quantity of antibody bound prolactin-^{131}I which occurs with increasing levels of 3 pituitary preparations and with 2 sera are shown in Fig. 1. B367 is serum from a rat bearing a prolactin secreting tumor, B329 is the purified preparation of rat prolactin, B351 serum and B351 pituitary were obtained from normal diestrous females, and B330 is a purified rat growth hormone (GH). The region of the curves where 20–80% of the prolactin-^{131}I was bound to the antibody was used for estimating relative potencies. In this area the curves for all preparations tested were similar in shape and slope. In all cases estimates of relative potency were made from duplicate tubes in at least 3 and usually 4 points on the dose response curve in each assay. The radioimmunoassay estimates of prolactin concentration were obtained by using the pituitary homogenate from normal females (B351, equivalent to 1.24 μg of NIH-P-B1/mg) as a standard preparation. This preparation was assigned

TABLE I. Prolactin Content of Rat Pituitary Preparations Prolactin Content.

Preparation	Bioassay	Radioimmunoassay	RIA range	RIA bioassay
B351[a]	1.24 (6)[c]	1.24[d]	—	1.0
B349[a]	0.80 (3)	0.60 (3)	(0.53–0.62)	0.75
B350[a]	1.33 (3)	1.62 (3)	(1.55–1.85)	1.22
B400[a]	0.70 (3)	0.39 (3)	(0.31–0.43)	0.56
B401[a]	1.00 (3)	0.60 (3)	(0.55–0.63)	0.60
B329(P)[b]	771.00 (3)	660.00 (3)	(639–687)	0.86
B330(GH)[b]		0.001 (1)		
B301(LH)[b]		0.0001(2)		
B341(FSH)[b]		0.0001(2)		

[a] Expressed in terms of NIH-P-B1 (μg/mg of wet wt.).

[b] Expressed in terms of NIH-P-B1 (μg/mg of dry wt.).

[c] Numbers in parentheses indicate number of assays.

[d] B351 was assigned its bioassay potency and used as standard for radioimmunoassay.

[e] Gay, U., Niswender, G. D., Midgley, A. R., Jr., and Reichert, L. E., Jr., Proc. Soc. Exptl. Biol. Med. (In preparation).

its bioassay potency in terms of NIH-P-B1 and all other preparations were compared to it in the radioimmunoassay.

Table I, shows the prolactin potency of pituitary preparations as estimated by bioassay and by radioimmunoassay. The ranges included for radioimmunoassay estimates are the extreme mean values obtained at all dose levels in all of the radioimmunoassays (at least 18 estimates). There was good agreement in all cases for estimates of prolactin potency. There was no prolactin detectable ($<.0001$ μg/mg) in purified preparations of rat luteinizing hormone (B301), or follicle-stimulating hormone (B341). The purified preparation of rat growth hormone (B330) had a prolactin potency of 0.001 μg/mg. These data suggest that rat LH, FSH, and GH do not influence the estimate of prolactin potencies in this assay. Further proof that pituitary hormones other than prolactin do not influence this assay was obtained by assaying the eluate from the various segments following polyacrylamide gel electrophoresis of a pituitary homogenate containing trace amounts of rat prolactin-^{131}I. Five-hundred μg of the homogenate were layered beneath the buffer on the surface of columns of 10.5% polyacrylamide gel (5 $\times$ 50 mm) and subjected to electrophoresis for 40 min with a 220 V gradient and a continuous buffer system of 0.011 *M* boric acid, 0.0032 *M* disodium EDTA and 0.077 *M* Tris at pH 8.9. Figure 2 depicts the elution profiles of LH, FSH, and prolactin from these gels. The LH (9) and FSH[2] content of the various segments was determined by radioimmunoassay. The prolactin content of each segment was determined with the radioimmunoassay described in this paper. The peak in prolactin activity was found well in advance of the peaks noted for LH and FSH and coincided with the area where prolactin-^{131}I migrated. These data again suggest that rat LH and FSH do not influence the quantitation of prolactin with this assay and further suggest that thyroid stimulating hormone (TSH) and GH do not influence the assay either, since TSH and GH have been shown to migrate between LH and FSH in a similar polyacrylamide gel system (10).

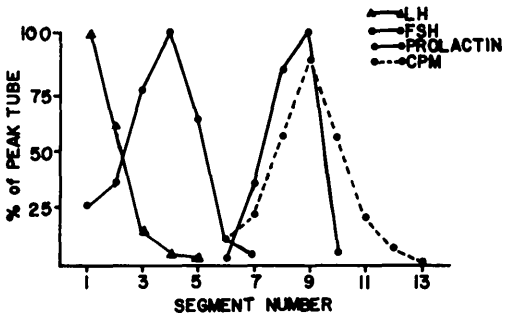

FIG. 2. LH, FSH, and prolactin content of the eluate following polyacrylamide gel electrophoresis of a pituitary homogenate. Each segment was 4 mm in length, with segment 1 beginning 2 mm from the origin.

TABLE II. Prolactin Levels in Serum from Rats in Different Endocrine States.

Endocrine condition	No. of animals	Prolactin content[a] Mean	Range[b]
Metaestrus (a.m.)	10	107	(58–218)
Diestrus (a.m.)	9	74	(50–112)
Proestrus (a.m.)	12	78	(26–117)
Proestrus (p.m.)	4	435	(317–640)
Estrus (a.m.)	9	194	(53–506)
Immature females (B400)	pool	12	
Mature males (B401)	pool	27	
Ovariectomized female (B349)	pool	20	
Ovariectomized female + 10 μg estradiol benzoate daily (B350)	pool	360	
Females with prolactin hypothalamic implants	5	84	(60–145)
Females with cholesterol hypothalamic implants	5	135	(75–228)
Prolactin secreting tumor (B367)	1	8000	

[a] Expressed in terms of NIH-P-B1 (mμg/ml).

[b] Range of values observed in each group.

Table II contains data regarding the prolactin levels in serum from rats in different endocrine states.

The highest levels of prolactin in serum obtained from rats during the estrous cycle occurred on the afternoon of proestrus, an observation which is in agreement with the data reported by Kwa and Verhogstad (11). In addition, there appeared to be a depression in serum levels of prolactin following stereotoxic placement of a prolactin implant into the median eminence when compared to rats implanted with cholesterol. This finding concurs with the observations of Clemens and Meites (12), who reported that implantation of prolactin into the median eminence of mature intact and ovariectomized rats resulted in increased hypothalamic content of prolactin inhibiting factor, reduced pituitary prolactin concentration and marked regression of the mammary glands. Finally, a rat bearing a prolactin secreting tumor had extremely high blood levels of prolactin equivalent to 8000 mμg of NIH-P-B1 per ml. In all cases the physiological data regarding prolactin levels obtained with this assay have agreed well with those reported in the literature using bioassay (13).

Bovine and ovine prolactin do not appear to cross react in this radioimmunoassay. Quantities of NIH-P-B2 and NIH-P-S8 as high as 1 μg/assay tube did not inhibit the reaction between the antirat prolactin and the rat prolactin-^{131}I.

It appears that a radioimmunoassay specific for rat prolactin has been developed which is capable of measuring the prolactin in as little as 100 μl of serum from an immature rat and in 10 μl obtained from most females on the afternoon of proestrus. This radioimmunoassay method is estimated to be about 1000–2000 times as sensitive as the local pigeon crop bioassay and has been used to measure serum levels of prolactin throughout the cycle, and in rats bearing prolactin secreting tumors or prolactin implants in the hypothalamus.

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Note added in proof. We have recently demonstrated that serum from hypophysectomized rats does not contain detectable prolactin.

1. Lyons, W. R., Cold Spring Harbor Symp. Quantitative Biol. 5, 198 (1937).

2. Arai, Y. and Lee, T. H., Endocrinology 81, 1041 (1967).

3. Bryant, G. C., Tech, B., Greenwood, F. C., and Linzell, J. L., Excerpta Med. Intern. Congr. Ser. 157, 76 (1968).

4. Kwa, H. G. and Verhofstad, F., Biochem. Biophys. Acta 133, 186 (1967).

5. Ellis, S., Grindelland, R. E., and Nuenke, J. M., Excerpta Med. Intern. Congr. Ser. 157, 77

(1968).
6. Reichert, L. E. and Midgley, A. R., Proc. Soc. Exptl. Biol. Med. 128, 1001 (1968).
7. Reece, R. P. and Turner, C. W., Missouri Univ. Ag. Expt. Sta. Bull. 266, (1937).
8. Niswender, G. D., Reichert, L. E., Midgley, A. R., and Nalbandov, A. V., Endocrinology, (in press).
9. Niswender, G. D., Midgley, A. R., Monroe, S. E., and Reichert, L. E., Proc. Soc. Exptl. Biol. Med. 128, 807 (1968).
10. Kragt, C. L. and Meites, J., Proc. Soc. Exptl. Biol. Med. 121, 805 (1966).
11. Kwa, H. G. and Verhofstad, F., J. Endocrinol. 39, 455 (1967).
12. Clemens, G. A. and Meites, J., Endocrinology 82, 878 (1968).
13. Meites, J. and Nicoll, C. S., Ann. Rev. Physiol. 28, 57 (1966).

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Studies on the Mode of Action of *N*-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide (MIH) (33658)

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N-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide (NIH) is one of a group of hydrazine derivatives that have been shown to be effective in the chemotherapy of experimental animal tumors (1,2). In man, MIH has been used specifically in the treatment of Hodgkin's Disease (3). Its exact mechanism of action is unknown, but the unaltered form is not thought to be biologically active (4) whereas, several of its possible metabolites may affect cellular metabolic processes. For example, in common with other hydrazine derivatives, MIH autoxidizes to produce hydrogen peroxide (5). It is possible that the intracellular generation of peroxide contributes to its cytotoxicity. We have previously described some of the metabolic consequences of the autoxidation of chemotherapeutic agents (6–8). Subsequent to autoxidation, isomerization and cleavage of the azo derivative of MIH may yield a variety of potentially toxic intermediates. These include aldehydes, other hydrazines, and methane. The formation of methane from the *N*-methyl group of MIH was demonstrated in rodents (9), and it is of special interest that this group also appears to be essential for biological activity (10). For these reasons, it was suggested that the transitory formation of methyl free-radicals may be intimately associated with the chemotherapeutic action of MIH (9).

Cytological studies revealed that MIH also exerts a marked influence on the mitotic cycle. In Ehrlich ascites carcinoma cells a suppression of mitosis brought about by a prolongation of interphase has been demonstrated (11). An outstanding feature of the cytological effects of MIH is the appearance of numerous chromatid breaks (11), and the treatment of cells with the drug over many transplant generations resulted in the formation of resistant lines (1). The present experiments were undertaken to explore the possible biochemical relationships between the chemotherapeutic actions of MIH and the development of resistance.

Materials and Methods. Ehrlich ascites cells were grown in male albino Swiss-Webster mice weighing from 21 to 25 g. Transplantation was carried out aseptically by collecting ascites fluid from mice after 8–10 days. The fluid was drawn into heparinized syringes and 0.2 ml (containing approximately 4×10^6 cells) was inoculated intraperitoneally into each receptor animal.

For *in vivo* experiments MIH was dissolved in isotonic saline just before use and injected subcutaneously into mice 6–8 days after inoculation of the ascites tumor. Cells were harvested from the peritoneal cavity by sacrificing the animals at appropriate time intervals after treatment with the drug. If