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Research Article

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Human Prolactin: Measurement in Plasma by In Vitro Bioassay

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ABSTRACT Prolactin has been measured in unextracted human plasma by a sensitive and specific in vitro bioassay. Secretory activity of breast tissue fragments from mid-pregnant mice, incubated in organ culture with human plasma, serves as the histologic end point. Sensitivity is 5 ng/ml (0.14 mU/ml) or somewhat better for ovine prolactin, and approximately 0.42 mU/ml for prolactin activity of human plasma at the dilutions used in the assay. Human growth hormone as it circulates in blood, like the material extracted from pituitary glands, is strongly lactogenic. Antisera to human growth hormone are capable of completely neutralizing the prolactin effect of large amounts (600 ng/ml) of human growth hormone added to the system. Plasma prolactin activity is less than 0.42 mU/ml in normal men and women. Of 26 patients with nonpuerperal galactorrhea, 14 had elevated prolactin activities ranging from 0.42 to 3.5 mU/ml. Growth hormone levels by radioimmunoassay were far too low, in general, to account for the observed prolactin activity. All of 14 nursing mothers, 1–30 days *post partum,* had elevated prolactin activity with a mean of 2.29 and a total range of 0.56–4.5 mU/ml. Growth hormone was in the low normal range in all of these subjects. Seven patients on psychoactive drugs of the phenothiazine series similarly had elevated prolactin activity with low growth hormone. Antiserum to human growth hormone, when preincubated with plasma samples from each of these three groups of subjects, produced no significant inhibition of prolactin activity. In two acromegalic patients with markedly elevated growth hormone levels, antiserum to growth hormone produced complete inhibition of prolactin activity in one and partial inhibition in the other. These studies indicate that human growth hormone and human prolactin are separate molecules, with little if any immunologic cross-reactivity, at least as demonstrated by the antisera used in this study, and that their release is governed by different physiologic mechanisms.

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INTRODUCTION

Prolactin has been actively investigated by endocrinologists for more than forty years, and has been found to exist in most vertebrate species (2). A number of physiologic properties, other than those associated with breast development, have been described (3). Chemical isolation of prolactin from several animal species has been accomplished. The amino acid sequence of ovine prolactin has been reported (4), and radioimmunoassays for rat (5, 6) and for ovine (7, 8) prolactin have been developed.

The relatively advanced state of these investigations in animals is in sharp contrast to the fragmentary knowledge that exists concerning prolactin in man. Strong lactogenic activity has been associated with highly purified human growth hormone preparations (9–11). Despite several attempts to isolate a separate human prolactin, no preparation in which growth hormone and prolactin activities are completely dissociated has yet been achieved (12–14), and the existence of a separate human prolactin has been seriously doubted (15). The problem is further complicated by the limitations of sensitivity and specificity of the conventional pigeon crop-sac assay when the latter is applied to human blood. We now report a sensitive in vitro bioassay, utilizing a mammalian target organ, which is capable of measuring prolactin in unextracted human plasma. The assay is described together with results of measurements in normal individuals and in those with various physiologic abnormalities. Evidence is presented which indicates that prolactin and growth hormone circulate as separate molecules in human plasma.

METHODS

Tissues used in this assay are taken from pregnant white Swiss mice of a local strain which are bred in this laboratory. The most satisfactory tissues come from nulliparous mice, aged greater than 10 wk, weighing between 25 and 35 g, at the eighth or ninth days of pregnancy. The animals are weighed daily both before and after exposure to a fertile male for a period of 24 hr. Weighings are done at the same time each day because of the considerable diurnal weight change observed in these mice. It has been found that seven days after mating, a weight gain of approximately 10% denotes pregnancy in about 80% of animals. After the mice have been killed by an overdose of ether vapor, the skin of the abdominal wall is reflected from a midline incision to expose the breast tissue. Thoracic and inguinal mammary glands are removed and put into a Petri dish containing Hanks' balanced salt solution[1] with added penicillin (250 U/ml) and streptomycin (250 μg/ml). Under a dissecting microscope, they are freed of excess connective tissue and transferred to a piece of filter paper presoaked in the above solution. Uncoated stainless steel razor blades[2] are used to cut blocks of tissue which measure approximately 2 × 2 × 1 mm. Three or four fragments, each from a different gland, are put on a square of lens paper on top of a stainless steel grid suspended in a plastic organ-culture dish[3] containing 1 ml of medium. The lens paper and grids are prewashed successively in water, 95% ethanol, and ether before heat sterilization. The dissection procedure is carried out as rapidly as possible. Each assay utilizes the mammary glands from only one mouse because of the considerable variation in response found between tissues from different animals of the same strain. The thoracic and inguinal glands together yield about 120 fragments. The tissues are incubated in the central compartment of the organ-culture dish, surrounded by approximately 5 ml of water to prevent evaporation of the medium. Cultures are maintained at 36°C in an atmosphere of 95% oxygen–5% carbon dioxide for 4 days. The fragments are then fixed in Bouin's solution, sectioned, and stained with hematoxylin-phloxine-safranine.

The incubation medium is commercial Medium 199[1] to which has been added pure beef insulin,[4] 10 μg/ml, hydrocortisone, 20 μg/ml, penicillin, 50 U/ml, and streptomycin, 50 μg/ml. In addition, human plasma is present in all dishes at a concentration of 30%. A large pool of human male plasma, with radioimmunoassayable growth hormone measuring less than 1 ng/ml, and no detectable prolactin activity, is kept frozen in small portions and thawed for use when needed. The plasma from this pool is used in all dishes containing standards. Ovine prolactin standard (NIH-P-S8, 28 U/mg)[5] is run in each assay at concentrations of 0, 1, 2, 5, 10, 20, and 50 ng/ml. Plasma samples from human subjects are routinely run at 30% and 10% final concentrations in the incubation medium; when necessary, further dilutions are made. The pooled male plasma is used to adjust the total plasma concentration in each dish containing a test sample to 30%. Each sample is run in at least two different assays; most samples reported here were assayed more frequently. Sterile technique was employed throughout, except that sterilization of plasma was not attempted. We have used the same method described, without added plasma, to test tissue and plasma extracts (16).

Each histologic section in every assay was examined and graded independently by two observers. Grading is done on an arbitrary scale from 0 to 4 plus, and the assessments of independent observers were in good agreement. A zero response, seen in the absence of prolactin, is characterized by the complete or nearly complete absence of red staining material from any of the lumina, together with low and immature appearing cytoplasm (Fig. 1). One plus is the smallest detectable degree of definite positivity, in which secretory material is present in small amounts in a minority (ca. 25%) of the lumina. Two and three plus are intermediate stages, with four plus representing a strongly positive response (Fig. 2). This picture, usually seen with prolactin at the 50 and sometimes also at the 20 ng/ml dose level, is characterized by abundant secretory material, staining darkly red, in virtually all lumina, with the cytoplasm having a mature secretory appearance. The score for each sample or standard represents the mean of both observers' scores for each of the three or four fragments present in the incubation dish.

Radioimmunoassays for growth hormone were done as previously described (17) using Wilhelmi growth hormone HS 1103C for standards and for radioiodination, and a rabbit antiserum to Raben growth hormone 13. Other antisera to various Wilhelmi growth hormone preparations have also been used for radioimmunoassay on some of these same specimens and have yielded essentially similar values.

RESULTS

Assay. A standard curve representing the combined experience of 44 assays is shown in Fig. 3. The addition of human male pooled plasma in concentrations of 30% to all standards increased the sensitivity of the assay. Table I compares certain features of the assay in the presence and absence of plasma. The increased sensitivity is displayed not only by increasing positivity at a given dose level, but by permitting the use of tissues taken from animals at an earlier stage of pregnancy. Tissues taken from animals less than 6 days pregnant have not, in our hands, been found sensitive enough to exhibit the typical histologic response expected when prolactin is added to the medium even at high concentrations. Likewise, tissues from animals more than 9 days pregnant have in general developed typically positive responses when plasma is added to the medium even in the absence of added prolactin. We have not considered tissues where this effect was present suitable for assay purposes. In assays run without human plasma, the mammary glands from animals up to 12 days pregnant have occasionally been adequate for use.

Sensitivity. Sensitivity varies considerably from mouse to mouse. With added plasma, prolactin is detectable in 91% of all assays at a concentration in the incubation medium of 10 ng/ml or 0.28 mU/ml, and in 22% of assays at a concentration of 2 ng/ml or 0.056 mU/ml ovine equivalents. Without plasma, prolactin at 10 ng/ml

[1] GIBCO, Grand Island, N. Y.

[2] Kindly provided by the Schick Corp., Milford, Conn.

[3] Falcon Plastics, Oxnard, Calif.

[4] Kindly supplied by Dr. Mary Root, Eli Lilly and Company, Indianapolis, Ind.

[5] Obtained from the Endocrinology Study Section, National Institutes of Health, Bethesda, Md.

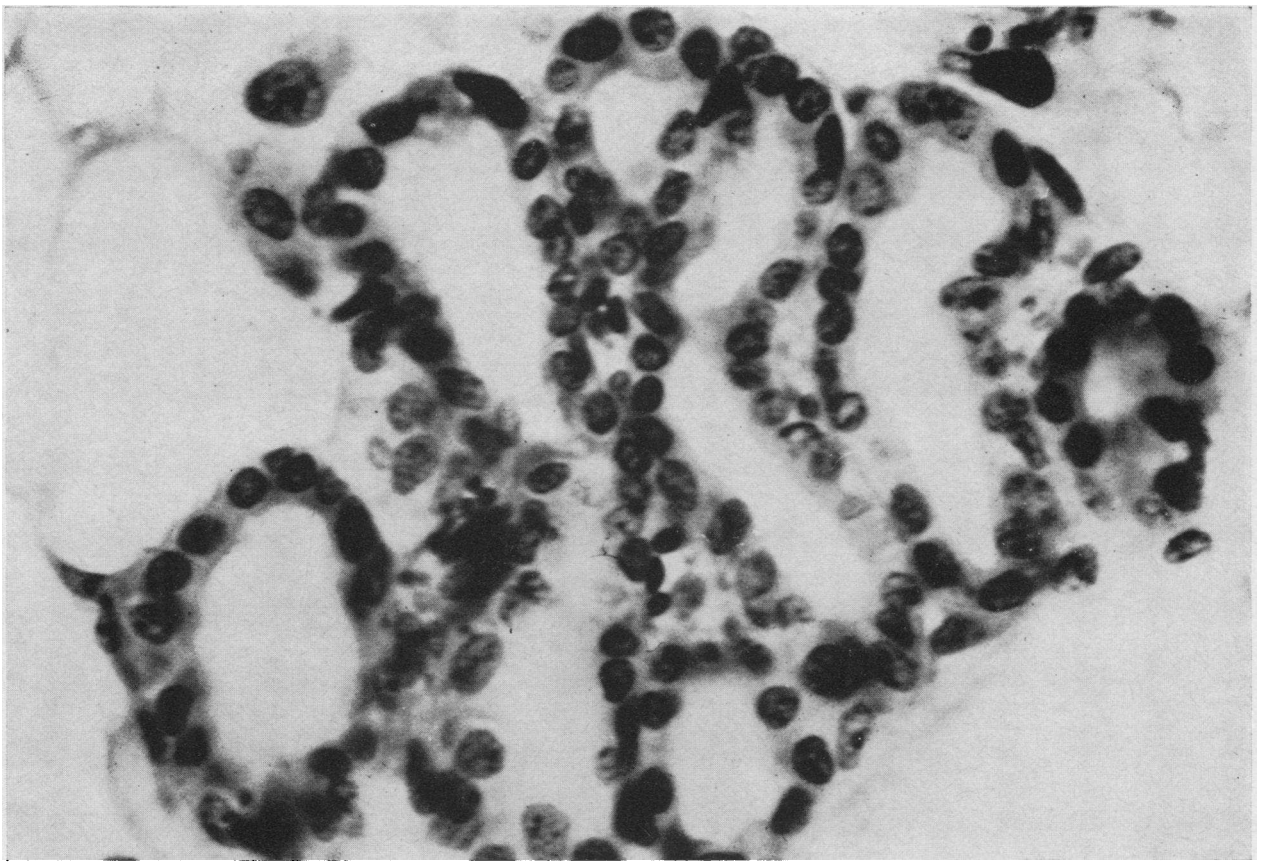

FIGURE 1 Mouse breast tissue after incubation without prolactin in the medium. Note the absence of intraluminal secretory material. × 430.

was detectable in only 31.5% of assays. The effective sensitivity in most assays is 5 ng/ml or 0.14 mU/ml. This corresponds to a sensitivity in human plasma, when the latter is present in the medium at 30% concentrations, of approximately 15 ng/ml ovine equivalents or 0.42 mU/ml. In occasional assays somewhat higher sensitivity, 6 ng/ml ovine equivalents or 0.17 mU/ml, can be achieved for plasma at 30% concentrations.

Specificity. The specificity of the assay has been investigated by the addition of a number of pituitary and nonpituitary hormones to the incubation medium (Table II). Of these hormones, only placental lactogen caused a positive response.[6] Estradiol at concentrations of 5 ng/ml and 2.5 μg/ml did not give positive responses, nor did it significantly affect the response to prolactin standards or to endogenous prolactin in the plasma of 10 patients when it was added to the incubation medium.

[6] Formal potency tests were not carried out on this substance, because the preparation available to us (Growth Factor of Placental Origin, Lederle Laboratories, Pearl River, N. Y. Lot 716049) was known to be impure.

Precision. The precision of the assay depends on the number of determinations and dose levels employed. Where large numbers of assays are carried out, as indicated by Fig. 3, satisfactory precision can be achieved. Single determinations at one dose level are inherently imprecise. All results on plasma samples reported in this paper are based on testing of the material in two or more dilutions, in at least two different assays, with the resulting mean thus representing at least four separate determinations. Because of the variations among different assays and the differing number of times various samples have been tested, no over-all figure for precision of results can be given. For most samples not at the limit of detectability of the assay, the standard error associated with the mean of all determinations is approximately ±30–35% when the sample is run in two assays, and ±25% when three assays are used.

Prolactin potency of human growth hormone. Several preparations of highly purified human growth hormone have been assayed for prolactin activity. All have been found to possess a relatively high degree of activity, ranging from approximately 50 to 75% of the ovine

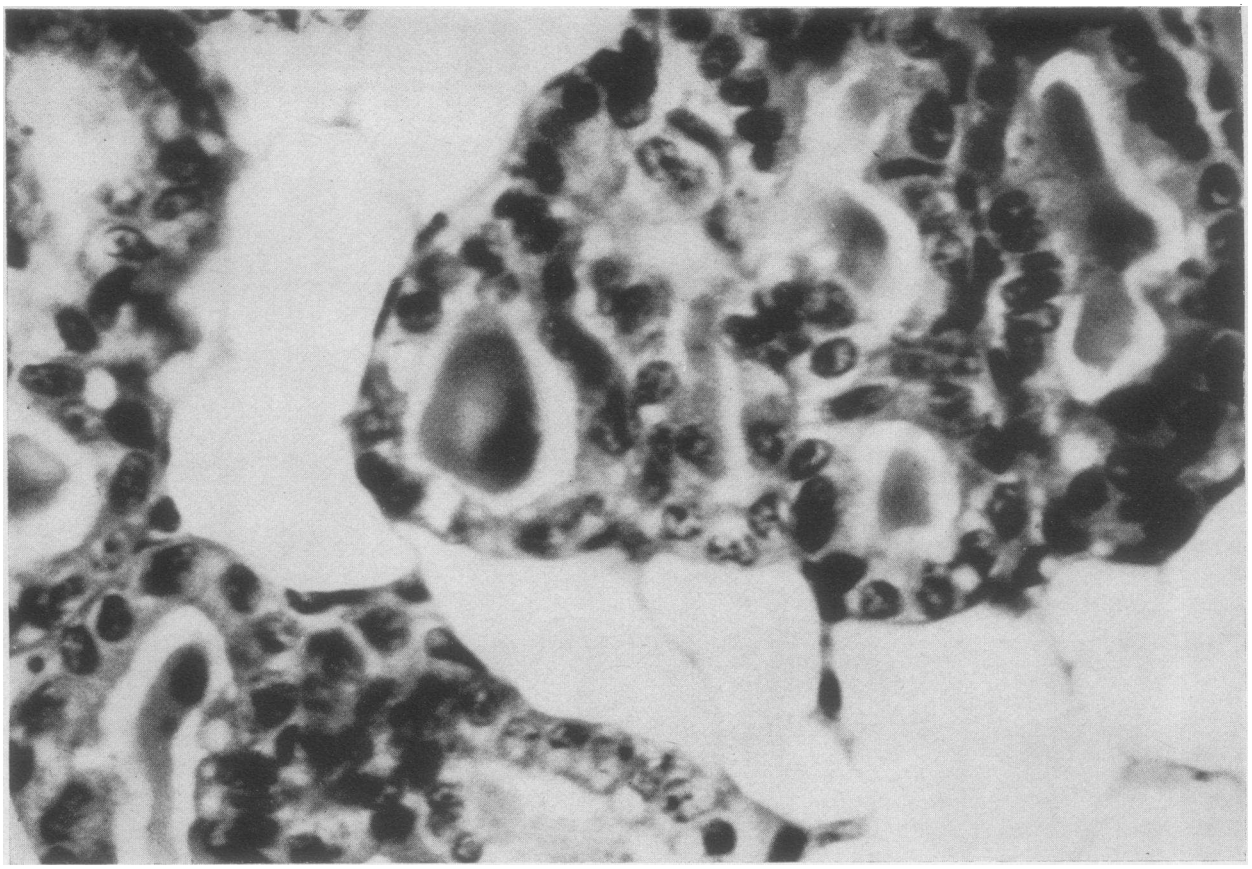

FIGURE 2 Mouse breast tissue after incubation with prolactin at a concentration of 50 ng/ml. Abundant secretory material is present in the lumina. × 430.

standard on a weight basis. The two most frequently assayed preparations have been Wilhelmi HS 1142 (growth hormone 2.84 IU/mg by the rat tibial line assay; prolactin potency 12.2 IU/mg by the systemic pigeon crop-sac assay)[7] and Wilhelmi HS 1103C (bioassay data not available; growth hormone potency identical to that of HS 1142 by radioimmunoassay). When dissolved in normal male plasma and assayed at several dilutions, the potency of HS 1142 was 16.0 U/mg with an SEM of ±4.20, and that of HS 1103C was 16.5 ±3.86 U/mg. Significant differences between the dose-response curves for growth hormone and ovine prolactin were not observed; the absence of such differences is a poor criterion of similarity in this assay, however, as compared with radioimmunoassay, because of the imprecise nature of individual observations. The somewhat higher prolactin potency of these growth hormone preparations by this assay, compared with what has been observed for these and similar preparations by the pigeon crop sac assay, is in accord with the observations of Forsyth, Folley, and Chadwick (18), who noted a similarly high prolactin potency for human growth hormone when the latter was tested in another mammalian system, that of the pseudopregnant rabbit.

Normal subjects. Assays for prolactin activity have been carried out on plasma samples from 40 endocrinologically normal subjects (Table III). There were 20 males ranging in age from 3 to 56 yr, and 20 females between the ages of 14 and 67. All of the males and all but one of the females had prolactin activities below 0.42 mU/ml. In the remaining female, prolactin activity was detectable at the threshold of sensitivity of the assay at 0.42 mU/ml (15 ng/ml in terms of the ovine standard). Plasma growth hormone on the same sample was 16 ng/ml. In two of the normal males who had growth hormone levels of 6.3 and 5.8 ng/ml, prolactin activity was also detected by unusually sensitive assays at 0.17 mU/ml.

Post partum subjects. Plasma samples from 14 nursing mothers, drawn 1–30 days *post partum,* have been assayed for prolactin activity and growth hormone lev-

[7] The growth hormone samples used in this work and the bioassay data were supplied through the courtesy of Dr. A. E. Wilhelmi.

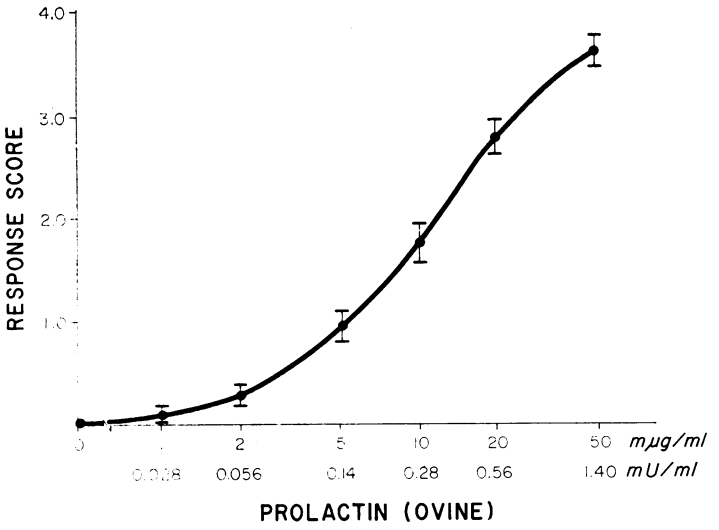

FIGURE 3 Dose-response curve representing the combined experience of 44 assays. Vertical bars indicate the SEM. Method of scoring is discussed in text.

els (Table IV). The interval between blood drawing and the previous episode of nursing varied, but in most cases was 2 hr or more. Prolactin activity was detectable in all plasmas, with a mean of 2.29 mU/ml and a total range of 0.56–4.5 mU/ml. Growth hormone concentrations were in the lower part of the normal range in all subjects, with a mean of 1.5 ng/ml.

Nonpuerperal galactorrhea. We have studied 26 patients with nonpuerperal galactorrhea (Table V). All of the patients except two (A. L. and U. W.) were referred to and examined by one of us. 14 of these patients had elevated plasma prolactin activity ranging from 0.42 to 3.5 mU/ml, together with normal growth hormone levels ranging from 0.3 to 2.6 ng/ml. Four patients (T. M., J. H., B. C., and M. A.) had growth hormone levels greater than 10 ng/ml together with elevated prolactin activity. One of these patients, B. C.,[8] the only male in the series, had acromegaly with very high levels of growth hormone. Galactorrhea occurred after the withdrawal of estrogens used in his treatment. The remaining eight patients all had plasma prolactin activity less than 0.42 mU/ml, together with normal growth hormone levels. All of these eight patients were assigned a diagnosis of idiopathic galactorrhea, indicating that no apparent cause or correlation for the galactorrhea could be found. Most of these patients had regular menses, while those with higher prolactin activity in general had amenorrhea. A fuller clinical report of these patients is in preparation.

Patients on psychoactive drugs. Elevated prolactin activity, together with normal growth hormone levels, has been detected in the plasma of each of seven patients whom we have studied who were under treatment with drugs of the phenothiazine series in high doses (Table VI). All patients had a diagnosis of schizophrenia, but none had any apparent endocrine abnormality before treatment. Duration of therapy varied from 2 wk to 18 months at the time of testing. Only two of the patients, A. E. and A. M., had galactorrhea. In two patients, M. M. and A. M., who were studied before as well as during therapy, prolactin was undetectable before treatment was begun. Chlorpromazine,[9] when added to the incubation medium in doses up to 50 ug/ml, did not affect this assay.[10]

Studies with antiserum to human growth hormone. Preparations of human growth hormone, as well as

TABLE I

Comparison of Assay with and without Added Human Plasma

	Incubated with 30% plasma	Incubated without plasma
Number of assays	52	26
Average length of mouse pregnancy—days	8.5	9.6
% positive responses with prolactin at 10 ng/ml	91%	31.5%

[8] This patient was made available for study through the kindness of Dr. Anne P. Forbes.

[9] Kindly supplied by Dr. R. C. Hoppe of Smith Kline & French Laboratories, Philadelphia, Pa.

[10] This work is being done in collaboration with Dr. R. N. Wharton of the Department of Psychiatry, and will be published separately in more extensive form.

TABLE II
Specificity of Assay

Hormone	Concentration	Response
TSH (bovine)*	25 mU/ml	0
ACTH (porcine)*	50 mU/ml	0
Growth hormone (bovine)‡	125 ng/ml	0
Oxytocin (synthetic)§	0.25 U/ml	0
Vasopressin (lysine)§	0.25 U/ml	0
Estradiol 17β‖	2.5 μg/ml	0
"	5.0 ng/ml	0
"	0.5 ng/ml	0
Progesterone‖	5.0 μg/ml	0
Human chorionic gonadotropin¶	10 U/ml	0
Testosterone‖	5 μg/ml	0
Human placental lactogen**	30 ng/ml	pos.

* Armour Pharmaceutical Co., Chicago, Ill.
‡ NIH-GH-B14, Bethesda, Md.
§ Sandoz Pharmaceuticals, Hanover, N. J.
‖ Calbiochem, Los Angeles, Calif.
¶ Ayerst Laboratories, New York.
** Lederle Laboratories, Pearl River, N. Y.

plasma samples from patients with nonpuerperal galactorrhea, *post partum* lactation, and acromegaly, have been assayed before and after exposure to antigrowth hormone serum. A highly purified growth hormone preparation, HS 612A, having a growth hormone potency of 1.7 IU/mg by the rat tibial line assay and a prolactin potency of 9 IU/mg by the systemic pigeon crop-sac assay,[7] was used for immunization in a rabbit. The animal was bled 2 wk after the third subcutaneous injection of hormone emulsified in Freund's adjuvant. Serum from this animal was capable of binding 60% of trace amounts of ^{131}I-labeled growth hormone at a dilution of 1:150,000, and more than 95% at a dilution of 1:1000. For neutralization experiments, a 1:10 dilution was used, in which 0.1 ml of antiserum was incubated with 0.9 ml of human plasma at room temperature for periods of 2 hr or more. The mixture was then bioassayed in at least two dilutions as described above. Parallel incubation of the same plasma in identical tubes without antiserum, or with normal rabbit serum at 1:10 dilution was always carried out, with subsequent bioassay in the same assay as the specimens with antiserum. Neither the rabbit antiserum itself nor the normal rabbit serum were found to affect the bioassay under the conditions employed; the potency of ovine prolactin at all dose levels was unaffected by anti-HGH at 1:10 dilution. The effect of anti-HGH was also tested on human growth hormone standard (HS 1103C) dissolved in pooled normal male plasma at concentrations of 100, 500, and 2000 ng/ml. After incubation, 0.3 ml of each of these mixtures, as well as others without antibody, were bioassayed.

The results of these incubations are shown graphically in Fig. 4. At no concentration of human growth hormone added to normal male plasma, including 2000 ng/ml (which represented 600 ng/ml in the final breast tissue culture medium) could any prolactin activity be detected in the specimens incubated with antibody. Extreme prolactin effects, greater than those of our highest ovine prolactin standard (50 ng/ml), were noted with the growth hormone in plasma without antibody at 500 and 2000 ng/ml (150 and 600 ng/ml in tissue culture medium). The results on five patients with galactorrhea (A. L., L. M., M. H., A. E., D. N.) and four patients with *post partum* lactation (E. O., S. R., B. H., M. S.), all incubated with and without antiserum, are also shown in Fig. 4. For the purposes of the figure, the prolactin values after incubation with antiserum

TABLE III
Plasma Prolactin Activity in Normal Subjects

Number	Age	Prolactin activity	Human growth hormone
		mU/ml	*ng/ml*
Males			
18	9–56	<0.42	mean: 0.97
			total range: < 0.3–2.5
1	31	0.17	6.3
1	3	0.17	5.8
Females:			
19	14–67	<0.42	mean: 2.4
			total range: < 0.3–8.4
1	25	0.42	16.0

TABLE IV
Nursing Mothers

Patient	Days	Human growth hormone	Prolactin activity
	post partum	*ng/ml*	*mU/ml*
A. R.	1	0.7	2.5
R. H.	1	3.6	2.3
E. O.	1	1.5	2.8
U. G.	1	2.8	3.4
G. W.	3	<0.3	4.5
S. R.	3	2.8	2.1
B. H.	3	1.8	2.7
G. B.	3	1.0	2.0
U. J.	3	2.1	1.2
B. W.	4	0.3	1.4
M. S.	5	2.1	2.7
M. P.	6	1.0	0.56
S. S.	6	0.3	2.5
M. H.	30	<0.3	1.4
	Mean	1.45	2.29
	Total range	(<0.3–3.6)	(0.56–4.5)

TABLE V
Nonpuerperal Galactorrhea

Patient	Sex	Age	Diagnosis	HGH	Prolactin
				ng/ml	*mU/ml*
A. L.	F	25	Chromophobe adenoma	1.1	3.5
L. M.	F	30	Pituitary tumor (not removed)	1.0	1.5
L. L.	F	29	Craniopharyngioma	0.8	0.56
T. M.	F	21	Chiari-Frommel syndrome	10.1	0.42
J. H.	F	25	Chiari-Frommel syndrome	10.5	0.42
M. H.	F	24	Chiari-Frommel syndrome + Fluphenazine	1.7	2.5
A. M.	F	52	Perphenazine + amitriptyline	0.3	2.1
A. E.	F	38	Perphenazine	0.3	3.1
D. N.	F	29	Oral contraceptive withdrawal	1.0	1.6
R. B.	F	30	Oral contraceptive withdrawal	1.7	0.84
G. G.	F	32	Oral contraceptive withdrawal	0.3	0.56
U. W.	F	49	Alpha methyl dopa	0.8	1.7
E. P.	F	56	Isoniazid	0.9	<0.42
B. C.	M	39	Acromegaly + estrogen withdrawal	180.0	2.1
J. H.	F	29	Hypopituitarism (sarcoid)	0.9	0.28
M. C.	F	30	Idiopathic	2.6	1.1
E. G.	F	40	Idiopathic	0.7	1.0
M. A.	F	28	Idiopathic	15.5	0.42
G. B.	F	30	Idiopathic	2.8	<0.42
J. M.	F	28	Idiopathic	0.4	<0.42
A. M.	F	27	Idiopathic	3.7	<0.42
R. U.	F	30	Idiopathic	1.0	<0.42
M. M.	F	22	Idiopathic	1.7	<0.42
G. S.	F	21	Idiopathic	0.9	<0.42
M. W.	F	40	Idiopathic	0.8	<0.42
M. P.	F	24	Idiopathic	5.0	<0.42

were expressed as percentages of the same plasmas without antiserum. The latter values are expressed as 100%. Mean prolactin activity without antiserum was 2.4 mU/ml (total range 1.5 to 3.5 mU/ml) for the galactorrhea patients and 2.6 mU/ml (total range 2.1 to 2.8 mU/ml) for the *post partum* subjects. Mean prolactin activity after incubation with antiserum was 124% of controls for the galactorrhea patients (individual values ranged from 68% to 159% of controls) and 92% for *post partum* subjects (individual values ranging from 55 to 155% of controls). Neither the group means nor any of the individual values differed significantly when results with and without antibody were compared in both the galactorrhea and *post partum* groups. All of the plasma growth hormone levels in these patients were in the lower part of the normal range (mean 1.0 and 2.1 ng/ml for the galactorrhea and *post partum* patients respectively).

Two patients with acromegaly have been studied in this manner (Fig. 4). One (R. H.), whose growth hormone was 105 ng/ml, had plasma prolactin activity of 11.2 mU/ml (±2.50 SEM) which decreased to 4.5 mU/ml (±1.03 SEM) after exposure to antiserum ($P <$ 0.05). The other patient (S. M.), with a growth hormone of 170 ng/ml, had plasma prolactin of 3.4 mU/ml (±0.81 SEM) which became undetectable (< 0.42 mU/ml) after incubation with antiserum.

More limited tests have been carried out with two other antisera prepared against highly purified growth hormone preparations (Wilhelmi HS 617B and HS 497C). Under identical conditions of incubation, no sig-

TABLE VI
Prolactin during Drug Therapy

Patient	Sex	Age	Drug	Dose	HGH	Prolactin activity
			wk	*mg/day*	*ng/ml*	*mU/ml*
C. G.	F	18	Fluphenazine (2)	6	<0.3	0.42
A. E.*	F	38	Perphenazine (78)	12	0.3	3.1
A. M.*	F	52	Perphenazine (12) + Amitriptyline (12)	12	0.3	2.1
D. V.	M	23	Chlorpromazine (4)	2000	0.5	1.7
M. M.	M	25	Chlorpromazine (4)	1500	3.7	0.56
T. B.	M	20	Chlorpromazine (8)	2000	0.4	0.84
A. J.	F	44	Imipramine (4)	150	1.1	0.56

* Galactorrhea.

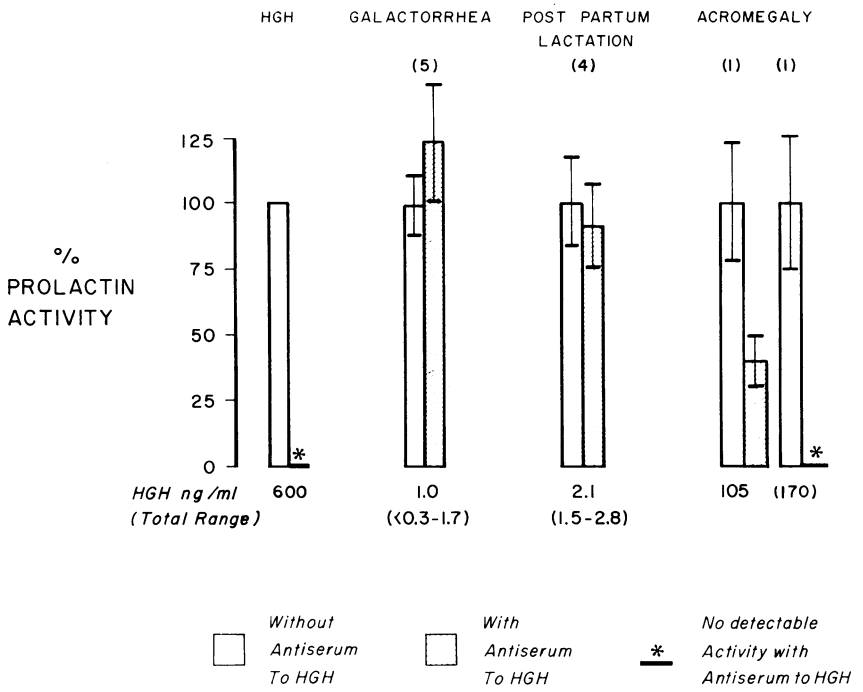

FIGURE 4 Prolactin activity with and without prior incubation of sample with antiserum to human growth hormone. Vertical bars indicate the SEM. The prolactin activity after exposure to antiserum (shaded bars) is represented as a per cent of the activity without such exposure (open bars). The latter are represented as 100%, with actual values for prolactin activities given in the text. Values for growth hormone (in specimens without antiserum) are indicated on the figure.

nificant differences between these antisera and antiserum to HS 612A were noted in their ability to neutralize the prolactin activity in the plasma of patient S. M. with acromegaly, or in the lack of ability to neutralize the prolactin activity in one patient with nonpuerperal galactorrhea and another patient with *post partum* lactation.

DISCUSSION

The major obstacle to progress in the field of human prolactin studies in recent years has been the lack of an adequately sensitive and specific bioassay. Of the many assays which have been developed, the most sensitive until recently have been the various local modifications of the Riddle and Bates original systemic assay (19) employing the pigeon crop epithelium as an end-organ. For assays utilizing a morphologic end point, threshold sensitivities have been reported of approximately 12 mU in a total volume of 0.4 ml injected material (14, 20), while for the more recent modification employing crop mucosa weight (21, 22), a sensitivity of 4 mU in 0.4 ml, or 10 mU/ml, can be achieved (22). Somewhat higher sensitivity has been claimed for a modification involving tritiated methylthymidine uptake (23), but full data on this assay have not yet appeared. Despite its utility in the measurement of pure hormone preparations and the relatively good precision of which it is capable, the pigeon crop-sac assay has not in general proved sensitive enough to measure prolactin in human blood without elaborate preliminary extraction procedures. The presence in both extracted and unextracted biological fluids of nonspecific substances causing inflammatory reactions can produce serious problems of specificity (14, 24, 25). An assay of higher sensitivity, employing a mammalian end-organ, is made possible by using mouse breast tissue in organ culture.

The observations of Hardy (26), Lasfargues (27, 28), and Elias (29), on the maintenance and differentiation of mouse mammary tissue cultivated in vitro, laid the basis for extensive further study of this system and definition of the hormonal requirements for cell differentiation and milk protein production (30–35). Prolactin, in the presence of insulin and hydrocortisone, has been found to be essential for the development and maintenance of full secretory activity. The actions of prolactin on the breast in organ culture have been sufficiently specific to suggest that the system could be used for assay purposes. Prop (36) proposed an assay, based on lobulo-alveolar development in glands from virgin mice (37), for which a sensitivity of 1 mU/ml was claimed. Full details of this assay have not as yet ap-

peared. This method has been discussed and criticized by Mishkinsky, Dikstein, Ben-David, Azeroual, and Sulman, who developed a somewhat similar assay using breast tissue from virgin rats; positive responses were seen in 80% of specimens at 10 mU/ml, in 50% at 1 mU/ml, and in 20% of controls without prolactin (38). Neither assay has, to our knowledge, been applied to plasma or other biological fluids.

Our own studies of cultured breast tissue from pregnant mice indicated that prolactin could produce morphological changes in this system at levels considerably below what had previously been reported, and that the method had advantages as a highly sensitive and specific bioassay (16). Initially, we believed that human plasma in significant amounts might be toxic to the cultures, and we explored a number of purification techniques for use in conjunction with the assay. Although plasma prolactin activity could be detected in a number of patients after extraction by a modification of the procedure of Canfield and Bates (39), the difficulties of all extraction methods and uncertainties as to losses prompted a more extensive investigation of the effects of whole plasma. From these experiments, it became evident that not only is the organ culture system tolerant of human plasma, but that the presence of the latter appears to stabilize it; the incidence of necrotic reactions is reduced, and the sensitivity for prolactin is increased. The limit of prolactin detectability in the final incubation mixture is now approximately 0.14 mU/ml for most assays, and occasionally as low as 0.06 mU/ml. Human plasma is currently used routinely in all assays, even those involving only highly purified materials.

That a system employing breast tissue in organ culture is capable of inherently high sensitivity is further shown by two reports which have very recently appeared. Forsyth (40) has reported as assay using explants of breast tissue from pseudopregnant rabbits in which prolactin can be detected at 1.2 mU/ml. Positive responses were reported in three patients with galactorrhea and three nursing mothers whose plasma was added to the assay mixture at 10% concentrations. In addition, Loewenstein, Mariz, Peake, and Daughaday have reported a bioassay employing mid-pregnant mouse breast tissue in organ culture in which N-acetyllactosamine synthetase activity, measuring radiochemically, is used as the end point (41). Sensitivity in this assay is comparable to what we have reported.

It should be noted that the advantages of a completely objective end point, employing radiochemical or other means, are considerable: freedom from possible subjective bias in interpreting the microscopic sections, the possibility of obtaining a more finely graded response, and a considerable saving in the time required to evaluate the histology of every section in a large assay. The very good agreement between independent scores by two investigators, however, when, as is always the case, the slides are read in a random order without the observer's knowledge of what samples they represent, encourages our belief in the validity of the scores. Furthermore, every score represents the mean of individual ratings on each of several fragments in the incubation dish; a wide range of values intermediate between fixed scoring levels is thereby made possible. Despite its disadvantages, moreover, the use of a morphologic end point carries with it one advantage of major importance in a bioassay such as this: ability to check by direct observation the functional state of every piece of tissue involved. On occasions, and in spite of the most careful technique, necrosis will appear; it may be limited to all of the fragments in a single dish, or some but not all of the dishes in an assay. It may appear in sample dishes without being present in those containing standards. Less often, fragments of a lymph node or other extraneous tissue will be included in a dish. Although these difficulties are not common, they could be a cause of considerable error in individual determinations if not recognized. Despite its inconveniences, direct microscopic examination of each tissue fragment seems to us to afford information about an assay and assurance as to its validity which can be obtained in no other way. These may be particularly important when complex biologic mixtures, rather than purified standards, are being assayed.

Previous attempts to measure prolactin activity in human blood by means of the pigeon crop-sac assay have yielded widely conflicting results; this subject has recently been thoroughly reviewed (14). In part, these discrepancies appear to have been due to the extraction procedures, acid-acetone methods having led to results which could not be duplicated (14, 42), and in part to nonspecific stimulation produced by the extracts themselves in the local crop-sac assay. Our own values for plasma prolactin in normal subjects (less than 0.42 mU/ml), in patients with galactorrhea (less than 0.42 mU/ml to 3.5 mU/ml) and in nursing mothers (0.56 to 4.5 mU/ml) are lower than any previously reported, though in the category of galactorrhea they show some overlap with those of Canfield and Bates (39). The differences between our values and those of others may be accounted for in large part by the elimination of artifacts due to extraction, plus the use of a mammalian end-organ in which nonspecific inflammatory responses do not mimic those of secretion.

Despite the difficulties which have been experienced in attempts to isolate a human prolactin separate from growth hormone (12–14), evidence from several sources has previously suggested that such a hormone does in fact exist: nonpuerperal galactorrhea in humans is not

usually associated with signs of acromegaly (39, 43). Growth hormone levels are not elevated in nursing mothers during the puerperium (44–46). Patients with idiopathic growth hormone deficiency are able to have normal *post partum* lactation (47). Histologic studies of pituitaries from pregnant women have long indicated the presence of "pregnancy cells" (48–50) which are difficult to observe in normal adult pituitaries. By special staining methods, similar appearing cells have been noted in pituitaries from *post partum* subjects (51, 52), and patients with galactorrhea (53, 54). High prolactin activity has been extracted from pituitary tumors of women with galactorrhea (54–56). The case of Peake, McKeel, Jarett, and Daughaday was particularly well studied by histologic as well as hormonal analysis, and it was further shown that this patient's tumor extract, which contained unusually low immunoassayable growth hormone, had prolactin activity which could not be neutralized by preincubation with antigrowth hormone serum (54). Organ cultures of human fetal pituitaries have been reported to show decreasing immunoassayable growth hormone liberated into the medium with time, along with increasing prolactin activity (57; similar studies by others have not been wholly in accord with this finding (58).

We have previously reported that individuals with elevated plasma immunoassayable growth hormone, due to insulin induced hypoglycemia, acromegaly, or other causes, regularly had detectable plasma prolactin activity; this activity, unlike that of patients with galactorrhea or *post partum* lactation, could be largely or completely neutralized with antigrowth hormone serum (59). These studies indicated to us that the growth hormone molecule in blood, like that extracted from human pituitaries, possessed intrinsic lactogenic activity. We have since become aware of a report by Pasteels (60), amplifying an earlier observation (61), indicating that the crop-sac activity of extracts of fetal pituitary cultures, of serum from two patients with nonpuerperal galactorrhea and of pooled *post partum* plasma, could be attenuated with antiserum to the culture extracts but not with antiserum to human growth hormone. All extracts were prepared by an acid-acetone method. Growth hormone in the human sera was not measured. Though the methods employed by Pasteels have not always yielded similar results in other hands, his conclusions about the separability of human growth hormone and prolactin appear to be justified. The careful morphologic observations of Pasteels on animals and human pituitaries (51) provided early support for this thesis, of which he has been a constant advocate, at a time when there was little but clinical evidence in its favor.

The studies presented here and in our earlier paper (59) indicate that human prolactin and human growth hormone, like their counterparts in lower animals, are separate molecules with little if any immunologic cross-reactivity. Some degree of cross reactivity may exist, but if it is demonstrated by other antisera, the possibility should be borne in mind that such antisera may contain antibodies to trace contaminants of human prolactin present in the growth hormone used for immunizing purposes. The growth hormone molecule in blood resembles that extracted from human pituitaries in possessing a high degree of prolactin activity. Its role as a lactogenic agent is probably normally insignificant; the low levels of growth hormone together with the high prolactin activity found in *post partum* subjects, as well as in those with galactorrhea, suggest that lactation is primarily governed by prolactin. An exception may possibly exist in the galactorrhea occasionally associated with acromegaly. Our studies in the acromegalic patients R. H. and S. M., as well as those reported previously (59), indicate that prolactin hypersecretion may accompany that of growth hormone in some acromegalic patients but not in others. The data also suggest the possibility that in acromegaly unusual molecules may circulate, either because of direct pituitary secretion or altered peripheral degradation, which have differing ratios of immunologic growth hormone activity to biological prolactin activity.

These studies also provide further evidence for the feasibility of a radioimmunoassay for human prolactin. Work is in progress toward this goal in this and other laboratories (62, 63); the very recent work of Friesen and his collaborators (63) provides exceptional promise in this direction.

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