monkeys was supplemented by the addition of trivalent and divalent chromium to the drinking water. The trivalent chromium supplementation produced an improvement in the tolbutamide and glucose tolerance responses of impaired monkeys when the drinking water was maintained at neutral pH but not at mildly acid pH. Normal monkeys supplemented with divalent chromium resulted in the appearance of impaired responses to tolbutamide and glucose tolerance tests.

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Relation of Mammotropes to Mammary Tumors VI. Immunoassays of Rat Prolactin and Growth Hormones* (32623)

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Experiments described in earlier communications (1–4) indicate that hormones of the pituitary mammotropes (Mt) play a determining role in the induction and progressive growth of mammary tumors of the rat. These cells are commonly called "acidophiles." It is generally believed that acidophiles can secrete both growth hormone (GH) and prolactin (P). A review of experimental data (5) led us to conclude that a single cell can secrete both types of hormones. Until recently these hormones were identified by bioassays only. More recently acrylamide gel electrophoresis led to recognition of the P and GH profiles (6). Immunoprecipitation (7) and radioimmunoassays (8) enable simple quantitation of rat GH and P.

Presently we shall describe the development of anti-rat prolactin (a. rP) sera, the immunologic distinctness of rat P and GH and the use of these antisera to quantitate P and GH in pituitary extracts and in organ cultures of pituitaries.

Materials and Methods. The purified rat GH was obtained from Dr. A. E. Wilhelmi (R 86567, containing 2 U/mg; designated rGH/W) and from Dr. S. Ellis (GH-II-23-B; designated rGH/E).

Purified rat P was obtained from Dr. R. Bates (RB 18-115C; 1.6 U/mg; designated P/B and from Dr. S. Ellis (XLVI-35C; designated P/E.).

Antisera were prepared by immunization of rabbits as described previously (7). The quantity of antigen used was kept close to the threshold in order to minimize antibody production by minor antigenic components present in the material injected into rabbits. For similar reasons the rabbits were exsanguinated as soon as a satisfactory precipitation was obtained with the antigen under study.

Organ cultures were set up according to the technique of Nicoll and Meites (9). Fragments of normal female rat pituitaries, enlarged pituitaries of rats given diethyl-

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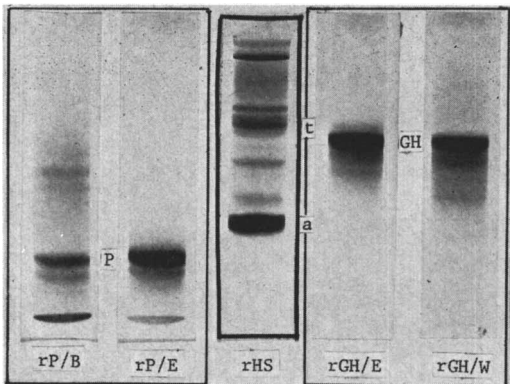

FIG. 1. Acrylamide electrophoresis of 100 μg each of two rat prolactins and two rat growth hormones; and three λ of a rat hypox serum. Abbreviations: a = albumin, t = transferrin, GH = growth hormone, P = prolactin.

stilbestrol (DES) pellets, and of mammotropic tumors (MtT/W15) were incubated in tissue culture medium 199. The culture fluids collected at 1–6 days, dialyzed, and lyophilized.

Pituitary extracts were prepared by the techniques of Jones *et al.* (6). The pituitary donor rats were thoroughly perfused with 0.9% saline and the pituitaries collected appeared pale, "bloodless".

Agar-gel precipitation. The procedure was similar to the one previously described (7) with the following modification. In order to block antibodies against normal serum proteins one drop of serum of hypophysectomized rats (HS) was added into the well containing impure antisera instead of absorbing such sera in the test tube with HS. This technique proved adequate to block the contaminating antibodies against normal serum proteins even when the titer of the latter exceeded that of the antihormones.

Acrylamide disc electrophoresis was performed according to the method of Ornstein (10) in 7.5% gel at pH 9.5, 2 mA.

Results. The acrylamide profiles of the rat GH and P used are shown in Fig. 1. Some contaminants of rGH/W and rP/B are indicated in these profiles. Nevertheless they were used for immunization because only these preparations were available in sufficient quantities. Of the more pure preparations of rGH/E and rP/E only small quantities were available; these were reserved for the precipitation tests. The major serum contaminants in these antisera were normal serum proteins which were blocked or absorbed with HS before use.

The results of cross precipitation tests of rP and rGH with a. rP/B and a. rGH/W (absorbed or blocked with HS) are shown in Table I. The data indicate that, within the range tested, these antisera were specific. The smallest quantity of antigen in this table can be considered to represent the threshold. These reactions occurred after 2–4 days; reactions with larger quantities of hormone appeared within 20 hours. Completeness of removal of antinormal serum proteins from the antihormone is indicated by the negative reactions with HS: Thus having obtained specific a. rP and a. rGH, the concentration of P and GH were tested in extracts and organ cultures of pituitaries.

In Expt. I enlarged pituitaries of female rats that had been treated with DES pellets, and in Expt. II normal female rat pituitaries were cultured. The fluids collected at 1, 3, and 6 days after incubation were dialyzed, lyophilized and assayed by precipitation with anti-rat prolactin (a.P), anti-rat growth hormone (a.GH) and antisera to sera of hypophysectomized rats (a.HS). This a.GH had no precipitins against normal rat serum. The a.P had to be absorbed with normal rat serum proteins (HS) after which it failed to precipitate normal rat sera but retained the anti-

TABLE I. Antigenic Specificity of Rat Prolactin and Growth Hormone.[a]

Antiserum to	Antigen μg	Prolactin B	Prolactin E	GH W	GH E	Hypox serum
rP/B	80					0
	25	+			0	
	2–5	+			0	
Organ	50–80	+		0	0	0
culture	25	+		0	0	
fluid of	5	+	+			0
DES pit.	2–0.5	+	+		0	
rGH/W	50	0				0
	25	0		+		
	10	0	0		+	0
	1			+	+	

[a] All antihormones were absorbed with HS.

TABLE II. Immunoprecipitation of Organ Culture Media of Pituitaries.

Collection days	μg of media	a.P[a] Exp. I	a.P[a] Exp. II	a.GH Exp. I	a.GH Exp. II	a.HS Exp. I	a.HS Exp. II
1	100+	++	++	++	+		+
	50–20	+	+	±	0	+	0
	10–5	+		0		0	
3	50	++	++				
	10	±	+	±	0		0
	2	0	0		0		
6	400				+		0
	100	+	+	0	0		
	20	±	+	0	0		
6	5000		++		+		0
(cells)	2500	+	+	+			
	500	+	+	0			
	1–200	0	+	0			

[a] Absorbed with HS.

prolactin precipitins. The results are shown in Table II. The smallest quantity of purified hormone recognized by these antisera under the conditions of such precipitation test was approximately 1 μg. Table II shows that both growth hormone and prolactin are released in these organ culture fluids in large quantities.

The acrylamide profile (Fig. 2) of the organ culture fluid (Expt. I) shows a wider band in the prolactin than in the growth hormone region. This is in agreement with the results of the precipitation tests. Organ cultures of male pituitaries similarly assayed by us (not shown in the table) gave higher precipitation titers with growth hormone than with prolactin.

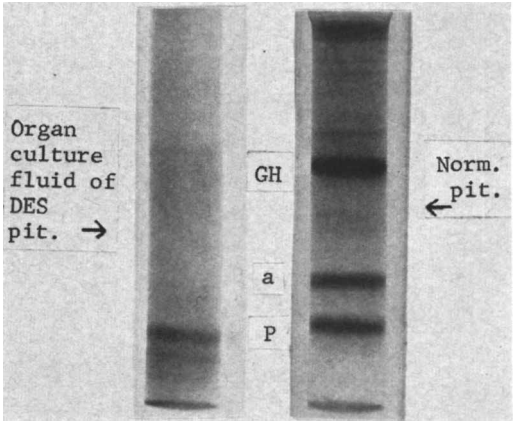

FIG. 2. Acrylamide electrophoresis of organ culture fluid of DES treated rat pituitary (1 mg) and of normal pituitary (1.8 mg).

When the organ cultures in Expt. II (Table II) were set up, pituitaries of rats of the same age and sex were extracted in phosphate buffer (pH 7.0) by the technic of Jones *et al.* (6). This extract contained 6.01 mg of protein per pituitary. Fourteen μg of this extract gave strong precipitates with both a.GH and a.P (but not with a.HS) while 1.4 μg failed to precipitate.

Antisera were prepared 3 years ago with organ culture fluids, pituitary and mammotropic tumor (MtT) extracts. It was found earlier that these sera gave strong reaction with the homologus antigens (7) but not until purified prolactin became available could the predominant antigen in these extracts be identified as prolactin. Earlier Nicoll and Meites (9) identified prolactin by bioassays as the predominant hormone in organ culture fluids of the pituitary.

Comments. Earlier data (1–5) indicated that hormones of pituitary mammotropes play a determining role in development of growth of mammary tumors of rats. The major hormones secreted by these cells are P and GH. The availability of specific antisera against rat P and GH enabled simultaneous tests for these hormones in various preparations derived from mammotropes. Further,

these antisera may aid in determination of the relative role of these two hormones in mammary tumorigenesis. A major therapeutic aim is to lower the level of mammary tumor stimulating hormones. In the course of therapeutic procedures these antisera may be useful in quantitation of P and GH in the sera of rats bearing mammary tumors. Variously hormone responsive transplantable mammary tumors can be readily obtained in the highly inbred W/Fu and Fischer rats.

Anti-P sera were first obtained by us in 1964 by immunizing rabbits with fluids of organ cultures of pituitaries from rats that had been treated with DES and with similar culture fluids of mammotropic tumors. Since the tumor strain used (W15) is strikingly GH-secreting, and is only moderately mammotropic it was expected that such antisera would precipitate GH. They precipitated the homologous antigen but not GH (7). This puzzling observation was cleared up with the availability of a. rP, which indicated the relatively rapid release of P in the organ cultures as is also indicated by bioassays (9). Furthermore it appears that P is more readily released than GH during extraction in neutral buffer, since antisera prepared with certain pituitary extracts produced predominantly a.P.

Recently Kwa *et al.* (11) prepared an extract of rat prolactin from the granular fraction of DES induced transplanted pituitary tumors by fractionation on DEAE column. With this preparation they produced a highly potent antiprolactin. Their findings parallel our organ culture studies. However, their antigens and antisera were not tested for GH and other contaminants. That our transplantable pituitary tumors, originally induced with DES, secrete growth hormone and prolactin (12) has been confirmed by Bates *et al.* (13), and MacLeod *et al.* (14). Some such tumors also secrete ACTH.

More data are needed on the relative antigenicity and precipitability of P and GH. The culture fluids used for immunization certainly contained minute quantities of other hormones, but the acrylamide gel electrophoresis indicated only two major components in the pituitary extracts: GH and P and only P in the culture fluid (Fig. 2). In acrylamide profiles of pituitaries, GH is usually more abundant than P. It deserves emphasis that the character of antisera do not faithfully indicate the spectrum of hormones in the material used for immunization. In the present study antibody production by minor antigenic components was minimized by keeping the amount of hormone extract used for immunization, close to the minimum needed for production of the wanted antibodies and the immunization period was short.

Summary. Anti-rat prolactin sera were produced in rabbits with organ culture fluids of pituitaries, mammotropic tumors and purified prolactin. Prolactin and growth hormone of the rat do not cross-precipitate with their respective antisera within a broad range of hormone concentration (1–50 μg) tested. Tests of organ culture fluids with antiprolactin and antigrowth hormone enabled quantitation of these hormones in pituitary extracts and organ culture fluids. The relative abundance of prolactin in organ culture fluid and in neutral buffer extracts of the pituitaries enabled the production of antiprolactin by short-term immunization using small doses of such fluids.

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Norepinephrine Levels in Various Areas of Rat Brain during Cold Acclimation* (32624)

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Norepinephrine (NE) released from peripheral sympathetic nerve endings is believed to function as a mediator for nonshivering thermogenesis in animals subjected to prolonged cold (1), and in the newborn (2,3). NE also occurs in the brain, within neurons (4), where its concentration in the hypothalamus is relatively high (5). The hypothalamus is known to contain thermodectors and to play an important role in the control of body temperature (6). Based on work with cats and dogs, Feldberg and Myers (7) have suggested that the release of hypothalamic stores of both NE and 5-hydroxytryptamine (5-HT) exert a controlling influence over the regulation of body temperature. If release of these amines from hypothalamic sites is necessary for central regulation of body temperature, changes in their hypothalamic levels might be expected to occur following exposure to cold. In previous work we (8) found no changes in hypothalamic or whole brain levels of NE or 5-HT following exposure of rats to cold of 2°C for up to 24 hours. However, following more prolonged exposure of rats to cold of up to 30 days, whole brain NE levels were found to increase late in the acclimation period, while 5-HT levels remained unchanged throughout.

The purpose of the present study was to determine whether the increase in NE previously observed in whole brain during prolonged exposure to cold (8) occurs selectively in the hypothalamus, or whether it also occurs in other brain areas of relatively high amine content, such as the midbrain and medulla oblongata.

Materials and Methods. A total of 108 male, Sprague-Dawley strain rats, obtained from Charles River Breeding Laboratories, and weighing between 130 and 175 gm, were used in this study. Half the group was exposed to cold of 1°C (0.5–3°C) in a cold room having a relative humidity of 70%. They were kept two per cage in metal cages having wire-mesh floors and fronts and measuring 8 inches wide, 7 inches high and 10 inches deep. The remaining group was housed in similar cages, two per cage, in air-conditioned animal facilities kept at a constant temperature of 25°C, with a relative humidity of 60%. Food (Purina Rat Chow) and tap water were always available *ad libitum*.

At 1, 15, and 30 days following exposure to 1 and 25°C, the body temperatures of equal numbers of both groups of rats were obtained by means of a thermistor probe inserted 4 cm into the colon and connected to a telethermometer. These measurements were taken while the animals remained exposed to either the 1 or 25°C environment. The animals were then immediately decapitated, their brains were removed, and the various brain areas were quickly dissected out for extraction and determination of NE. The extraction method for NE was a modification of that of Vogt (9) and its chromatographic separation and determination on the blood pressure of the pithed rat was a modification of the method of Crawford and Outschoorn (10). The "hypothalamus" was removed from the brain by a circular cut following the Circle of Willis, 3–4 mm deep, extending partly into the thalamus, and contained the major hypothalamic nuclei, the optic chiasma (rostrally) and the mammillary bodies (caudally). The "midbrain" was removed by cuts midway across the pons and cerebral peduncles and included both superior and inferior colliculi. The "medulla"

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