A Trypsin Technic for the Preparation of Isolated Rat Anterior Pituitary Cells (34520)

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(Introduced by M. N. Levy)

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Our knowledge of the secretion and action of hormones has been derived in large measure from studies on the whole animal, the perfused organ, the tissue slice, and the tissue homogenate. Isolated cells of the endocrine glands, or of the target tissues, represent a new approach to such studies. These preparations retain the advantages of the intact cell, yet eliminate problems of penetration of diffusion barriers inherent in the tissue slice. A suspension of isolated adipocytes as prepared by the collagenase technic of Rodbell (1) has proven to be a most fruitful test object for an examination of the actions of insulin. Swallow and Sayers (2) have devised a trypsin technic for the dispersion of cells of the adrenal cortex. These isolated cells respond to exceedingly small quantities of ATH with increased production of corticosteroid. We now wish to report the application of the trypsin technic to the isolation of cells of the rat anterior pituitary.

Materials and Methods. Dispersion of anterior pituitary cells. In each experiment, 6 to 12 male rats of the Sprague-Dawley strain, 250 to 450 g, were decapitated, and the anterior pituitaries were removed, quartered, and placed in a siliconized 50-ml Erlenmeyer flask with 20 ml of a Krebs-Ringer bicarbonate buffer containing 0.2% glucose (KRBG) and 0.25% trypsin (Worthington Biochemical Corporation). The KRBG was previously gassed for 10 min with a 95% O_2:5% CO_2 mixture and adjusted to pH 7.35 to 7.40. The pituitary tissue was mechanically agitated for 20 min by a siliconized glass paddle driven by a constant speed motor at 500 rpm (Waco Supreme Power Stirrer, Wilkens Anderson), while kept at 37° under a 95% O_2:5% CO_2 atmosphere. At the end of the 20-min period, the supernatant containing dispersed cells was drawn off with a siliconized pasteur pipette and placed in a siliconized, iced, 250-ml Erlenmeyer flask. An additional 20 ml of KRBG containing trypsin was added to the tissue, and the mechanical agitation was continued for another 20 min. After three such dispersion periods, the combined cell suspensions were placed in two siliconized 50-ml centrifuge tubes and centrifuged at 100*g* (gradually accelerated to speed during a 10-min period) for 30 min at 4°. The supernatant was aspirated, and the cells were resuspended to a concentration of one pituitary per ml in KRBG containing 3% bovine serum albumin (Sigma) (KRBGA) and 32 mg of lima bean trypsin inhibitor (LBI, Worthington Biochemical Corporation) adjusted to pH 7.35 to 7.40.

An aliquot of the cell suspension was combined with an equal volume of methylene blue (Neissers Stain A) (3), and the number of cells was determined in a hemocytometer. In some experiments, a portion of the cell suspension was extracted with an equal volume of 0.1 *N* HCl–0.9% NaCl (acid-saline), at 100° for 5 min. The extract was cooled and centrifuged, and, following appropriate dilutions, ACTH activity was determined by the method of Swallow and Sayers (2).

Incubation of anterior pituitary cells. Two methods of incubation were employed. In the first, pituitary cells were dispersed, centrifuged, and resuspended in KRBGA with LBI, as described above. Aliquots of 0.9 ml

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of this suspension were added to siliconized, 25-ml Erlenmeyer flasks. To each of these was added 0.1 ml of KRBGA containing the test substance [hypothalamic median eminence (HME) extract, cerebral cortical extract, or highly purified arginine vasopressin] or 0.1 ml of KRBGA alone for controls. The suspensions were incubated for 20 min at 37° under 95% O_2:5% CO_2 in a Dubnoff metabolic shaker (66 oscillations/min). At the end of the incubation period, the cells were removed by centrifugation at 100*g* for 30 min at 4°, using plastic or siliconized glass centrifuge tubes. An aliquot of the supernatant was diluted with an equal volume of acid-saline and heated for 5 min at 100°. Appropriate dilutions were made, and the ACTH content determined by the method of Swallow and Sayers (2).

In the second incubation method, the pituitary cells were resuspended to a concentration of two pituitaries per ml in KRBGA without LBI. Aliquots of 0.5 ml of the suspension were placed in the incubation flasks and preincubated for 10 min at 37° under 95% O_2:5% CO_2 with shaking (66 oscillations/min). Between 4 and 8 mg of LBI was added to the flasks in 0.5 ml KRBGA and the cells preincubated for an additional 5 min. KRBGA (0.1 ml) containing test substance or 0.1 ml of KRBGA alone for controls was then added, and the cells were incubated for 20 min. The cells were removed by centrifugation at 600*g* for 5 min at room temperature. Aliquots of the supernatant were taken and the ACTH activity was determined as previously described.

Preparation of test substances. An acetone powder was prepared from HME tissue collected from 68 male rats. The dried powder was homogenized in 10 ml of 0.2 *N* acetic acid, and sufficient 0.2 *N* acetic acid was added to give a volume of 34 ml. The mixture was refluxed for 10 min at 100°, cooled, and centrifuged for 1 hr at 16,000 rpm in the SS34 rotor of a Sorvall ultracentrifuge. The supernatant was decanted, brought to a volume of 34 ml with 0.2 *N* acetic acid, and stored at −20° C. These procedures have previously been described by Royce and Sayers (4).

An acetic acid extract of rat cerebral cortical tissue was prepared by the same procedure. The final volume of the extract was adjusted so that each ml was derived from a wet weight of cortical tissue equivalent to the wet weight of two rat HME.

Arginine vasopressin had a potency of 320 units/mg [pressor assay (5)].

Results. Mechanical agitation of rat pituitary quarters in the presence of trypsin led to the dispersion of isolated pituitary cells. The number of cells was variable, ranging from 650,000 to 1,440,000 per pituitary. Examination under the light microscope revealed most of the cells to be present as single, isolated cells, or in groups containing from two to six cells. A few large clumps of cells were present; these were excluded from the cell count listed above. In eight experiments, the ACTH content of the cells ranged from 2.3 to 5.2 milliunits per 100,000 cells.

The results of six experiments, in which isolated pituitary cells were incubated either alone (control incubates) or in the presence of various test substances, are presented in Table I. In each case, the incubation medium, separated from control incubates, contained significant amounts of ACTH (Table I, expt. 1a, 2a, 2b, 3a, 4a, 5a, and 6a). This activity was considerably reduced when the cells were preincubated for 10 min prior to the addition of LBI and harvested at 600*g* for 5 min at room temperature at the end of the incubation period (Table I, expt. 4a, 5a, and 6a). The addition of an acetic acid extract of HME at doses from 0.01 to 1.0 HME increased the ACTH content of the incubation medium (Table I, expt. 1c, 1d, 2c, 2d, 3d, 3e, 5c, and 6b). An acetic acid extract of rat cerebral cortex or 100 milliunits of highly purified arginine vasopressin had a similar, but somewhat smaller effect (Table I, expt. 3c, 5b, and 3b, respectively).

Discussion. The technic reported here for the preparation of isolated rat pituitary cells is similar to that previouly reported by Swallow and Sayers (2) for the preparation of isolated cells from rat adrenal cortex. Mechanical agitation of pituitary tissue in the presence of the enzyme trypsin is highly effective in dispersing large numbers of pitui-

tary cells. The functional and structural integrity of the cells prepared by this treatment is supported by several observations: (1) Under the light microscope, the cells appear to be intact and undamaged; (2) Electron micrographs, prepared by Dr. Sasha Malamed of Rutgers University, revealed the isolated pituitary cells to be membrane bound and to contain a rich supply of subcellular organelles, including secretory granules; (3) The isolated cells retain ACTH against a large concentration gradient; *i.e.*, whereas the isolated cells contain 2.3 to 5.2 milliunits of ACTH per 100,000 cells, the dispersion medium, KRBGA plus trypsin, contains only trace (Microunit) amounts of the hormone; (4). In a related series of experiments, incubation of isolated pituitary cells with radioactive amino acids was found to lead to the production of highly labeled trichloroacetic acid precipitable protein.

The medium separated from isolated pituitary cells incubated at 37° for 20 min contained significant amounts of ACTH. Under the conditions of our experiment, the ACTH content of the incubation medium is equal to

TABLE I. The ACTH Content of the Incubation Medium Separated from Isolated Pituitary Cells.[a]

Exp. no.	Experimental condition	ACTH content of incubation medium: As microunits/100,000 cells incubated	ACTH content of incubation medium: As % of control
1[b] a	Control	126 (124, 137, 117)	100
b	0.001 HME	120 (126, 114)	95
c	0.01 HME	156 (149, 152, 166)	124
d	0.1 HME	236 (244, 210, 254)	187
2[b] a	Control	131 (132, 130)	100
b	Control	131 (136, 126)	100
c	0.1 HME	196 (197, 197, 193)	150
d	0.1 HME	262 (252, 275, 259)	200
3[b] a	Control	125 (118, 132)	100
b	100 mU arg-vasopressin	200 (196, 204)	160
c	0.2 Cortical units[c]	173 (181, 164)	138
d	0.2 HME	266 (265, 267)	213
e	1.0 HME	357 (364, 349)	286
4[d] a	Control	22 (21, 23)	100
5[d] a	Control	17 (17, 17)	100
b	0.2 cortical units	22 (21, 23)	129
c	0.2 HME	38 (37, 39)	224
6[d] a	Control	58 (52, 63, 60)	100
b	0.2 HME	140 (140, 146, 135)	241

[a] The ACTH activity of the medium was determined at two or three dose levels (*i.e.*, dilutions) by the method of Swallow and Sayers (2); average values and the individual estimates, in parentheses, are presented.

[b] In exp. 1–3, dispersed pituitary cells were resuspended in KRBGA containing LBI, incubated at 37° for 20 min, and the incubation medium was separated from the cells by centrifugation at 100*g* for 30 min at 4°.

[c] A cortical unit is taken to be equivalent to an acetic acid extract of a weight of rat cerebral cortex equal to the weight of a rat HME.

[d] In exp. 4–6, dispersed pituitary cells were resuspended in KRBGA and preincubated for 10 min prior to the addition of LBI. After an additional 5 min, test substance (or, in the case of controls, KRBGA) was added, and the cells were incubated for 20 min. The incubation medium was removed by centrifugation at 600*g* for 5 min at room temperature.

the sum of the extracellular ACTH in the cell suspension added to the flasks, the ACTH released from the cells during the 20-min incubation period, and the ACTH released from the cells during centrifugation following incubation. Under control conditions, *i.e.*, in the absence of test substances, the medium contained a maximum of 131 microunits ACTH/100,000 cells. Since the cells contained 2.3 to 5.2 milliunits ACTH/100,000 cells, the amount of ACTH in the medium represents only 2.5 to 5.7% of the cellular content of the hormone. When the cells were preincubated at 37° for 10 min prior to the addition of LBI and separated from the incubation medium by centrifugation at 600*g* for 5 min at room temperature (Table I, expt. 4a, 5a, and 6a), the ACTH content of the medium from control incubates was considerably reduced. Although direct evidence is presently not available, we are inclined to explain this decrement in the following way: preincubation in the absence of LBI allows residual trypsin, carried over from the dispersion fluid, to inactivate extracellular ACTH present in the flasks prior to the start of incubation; centrifugation at room temperature results in the release of less ACTH than centrifugation at 4°. This latter view is supported by the finding in a single, preliminary experiment that exposure of pituitary cells to 0° for 20 min produced approximately a twofold increase in the ACTH content of the medium.

The addition of an acetic acid extract of HME or cerebral cortex, or of arginine vasopressin to the incubation flasks increased the amount of ACTH in the medium. This could be due to an increased release of ACTH and/or a decreased inactivation of ACTH in the medium. That is, HME, cortex, or vasopressin might bring about an increase in the ATCH content of the medium by acting as a corticotropin releasing factor (CRF). However, our findings, as well as those of others using *in vitro* bioassays to evaluate potential CRF's, might also be explained by postulating that these substances compete with ACTH for inactivating enzymes [see Barrett and Sayers (6) for a discussion of this problem] We are currently engaged in investigating these possibilities.

Summary. Isolated rat anterior pituitary cells have been prepared by mechanical agitation of quartered glands in the presence of trypsin. The cells appear morphologically and functionally intact and contain large amounts of ACTH. The medium separated from cells incubated at 37° for 20 min contains small but significant amounts of ACTH. Acetic acid extracts of rat HME or cerebral cortical tissue and arginine vasopressin increase the ACTH content of the medium.

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