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A Technic for the Preparation of Isolated Rat Adrenal Cells (33789)

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Two methods have been described for the preparation of isolated rat adrenal cells. Kloppenborg *et al.* (1) reported that the cells of the rat adrenal can be dispersed by incubating tissue fragments in a buffer which contained collagenase. The method is simple but suffers from a lack of reproducibility, probably due to variations in the potency or the purity of different batches of collagenase. Halkerston and Feinstein (2) employed a combination of enzymes. Rat adrenal quarters were preincubated in a medium containing trypsin, DNase and RNase, washed with a solution of trypsin inhibitor and then incubated in a medium containing collagenase and hyaluronidase. We have found that trypsin alone can effect the dispersion of adrenal cells. Isolated cells, so prepared, produce corticosterone in response to the addition of minute quantities [10 microunits (μU)] of ACTH.

Methods. In each of 5 experiments, 16 male rats, 150–450 g, of the Sprague-Dawley strain were anesthetized with ether starting between 8 and 10 a.m. The adrenals were removed, trimmed of fat and quartered. The 128 quarters were placed in a 50-ml Erlenmeyer flask containing 20 ml of cold (4°) Krebs–Ringer bicarbonate buffer to which had been added glucose (0.2%) and trypsin (TRL, Worthington Biochemical Corporation, Lot Nos. 800, 8CA and 8GA) (0.25%). The tissue was incubated at 37° and gassed with a mixture of 95% oxygen, 5% carbon dioxide. The incubate was stirred with a Teflon covered, hexagonal magnet, length 4 cm, width 1 cm (100 ± 20 rpm). At the end of each of five 20-min incubation periods, buffer containing dispersed cells was transferred to an iced 250-ml Erlenmeyer flask. An additional 20 ml of Krebs–Ringer bicarbonate buffer with trypsin was added to the adrenal quarters and the incubation continued. The pooled harvests of dispersed cells were centrifuged (4°) at 100*g* for 30 min (acceleration to final speed was gradual and occupied 10 min). The supernatant buffer was decanted and the cells were suspended in 30 ml of Krebs–Ringer bicarbonate buffer to which had been added glucose (0.2%), bovine albumin (Sigma, Lot 38, B-2170) (3%) and lima bean trypsin inhibitor (LBI,

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Worthington Biochemical Corporation, Lot No. 8FA) (2 mg/ml).

Aliquots of 0.9 ml of this cell suspension were placed in 25-ml Erlenmeyer flasks. To each was added, in a volume of 0.1 ml, a solution of ACTH or in the case of controls, vehicle. ACTH was the USP standard which is the Third International Standard (1962). Each vial contains 5 Subcutaneous Units or 1.5 Intravenous Units (3). The quantities of ACTH employed in this study are expressed in Intravenous Units. A solution of sodium chloride (0.9%) and bovine albumin (0.5%) was adjusted to pH 3.5 with 0.1 *N* HCl in 0.9% sodium chloride; ACTH was dissolved in this vehicle.

The flasks were incubated in a Dubnoff shaker (66 oscillations/min at 37°) in an atmosphere of 95% oxygen, 5% carbon dioxide. On the basis of time studies, 2 hr was chosen as the best period of incubation.

The quantity of corticosterone in each incubate (cells plus medium) was measured by the fluorescent method of Silber *et al.* (4).

To a 1.0-ml aliquot of cell suspension was added 1.0 ml of a solution of methylene blue (Neisser's Stain A) (5), and the cells were counted in a hemocytometer. The cells were spherical and contained a variable number of large lipid droplets. For experiments reported, cell counts ranged from 200,000 to 340,000/ml exclusive of cells in clumps and from 250,000 to 400,000/ml when the count included cells in clumps (2–20 cells/clump). These observations indicate that at least 75% of the cells in the suspension are single isolated cells.

Results. The results of 5 experiments are presented in Table I. Cell suspensions incubated without ACTH produced small but significant quantities of corticosterone. Addition of ACTH increased corticosterone production by the isolated cells. In Expts. 1, 2, 4, and 5 the relationship between corticosterone production and log dose of ACTH (10, 40, and 160 μU) was a straight line. In Expt. 3, the relationship was rectilinear between doses of 20 and 160 μU of ACTH.

In the absence of trypsin inhibitor, ACTH did not increase corticosterone production

TABLE I. Corticosterone Production by Isolated Adrenal Cells: Effect of Addition of ACTH.[a]

	ACTH (μU/ml)							
Expt. no.	0	5	10	20	40	80	160	320
1	0.19		0.57		2.70		4.55	
	0.18		0.35		2.00		4.55	
			(0.27)[b]		(2.16)		(4.36)	
2	0.19		0.88		2.75		3.57	
	0.13		0.78		2.38		3.13	
			(0.67)		(2.40)		(3.19)	
3	0.06	0.06	0.11	0.18	0.83	1.77	1.98	2.34
	0.08	0.07	0.10	0.41	1.10	1.64	2.36	2.34
	0.07	0.06	0.13	0.41	1.03	1.66	2.32	2.19
	0.06	0.08	0.12	0.39	1.08	1.60	2.34	2.34
		(0.00)	(0.06)	(0.28)	(0.95)	(1.61)	(2.18)	(2.23)
4	0.10		0.65		2.65		4.90	
	0.09		0.60		2.75		4.50	
			(0.53)		(2.60)		(4.60)	
5	0.04		0.29		1.60		2.82	
	0.04		0.27		1.31		2.84	
			(0.24)		(1.42)		(2.77)	

[a] Corticosterone production expressed as micrograms per 2 hr of incubation.

[b] Values in parentheses are averages for net corticosterone production (incubate plus ACTH minus control incubate).

TABLE II. Effect of Trypsin Inhibitor on Corticosterone Production by Isolated Adrenal Cells.[a]

Trypsin inhibitor (mg/ml)	ACTH (μU/ml)			
	0	10	40	160
0	0.13	0.13	0.14	0.13
	0.13	0.13	0.14	0.13
0.2	0.13	0.43	1.70	3.08
	0.13	0.42	1.73	2.70
2.0	0.19	0.88	2.75	3.57
	0.13	0.78	2.38	3.13
4.0	0.25	0.68	2.00	2.63
	0.20	0.63	1.98	3.00

[a] Corticosterone production expressed as micrograms per 2-hr incubation.

(Table II). The most likely interpretation is that in the absence of inhibitor, trypsin destroys ACTH. The results in Table II indicate that a concentration of 2 mg/ml of inhibitor is more effective than 0.2 or 4 mg/ml. In another experiment, 2 mg/ml was shown to be more effective than 0.5, 1.0, or 1.5 mg/ml.

Time studies were undertaken to determine the optimum period of incubation (Fig. 1). The results suggested that 2 hr was the most practical, i.e., production of corticosterone was sufficiently high to be estimated at 10 μU of ACTH; further incubation did not result in a large enough additional increase in output to warrant incubation for 4 hr.

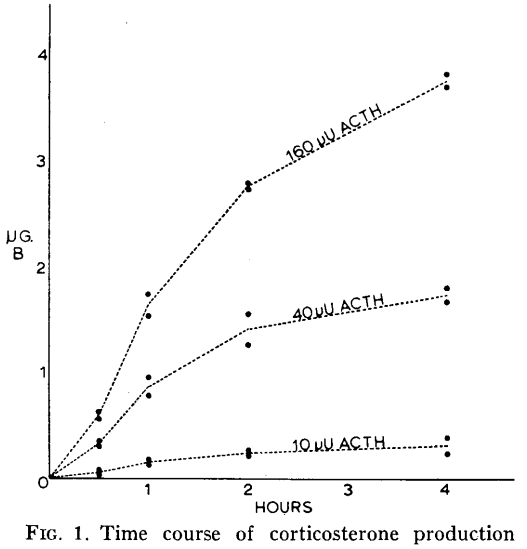

FIG. 1. Time course of corticosterone production by cell suspensions which contained 3 different doses of ACTH (10, 40, and 160 μU).

Discussion. The method for the dispersion of adrenal cells is simple and reproducible. Only one enzyme, trypsin, is required and the number of manipulative steps has been reduced to a minimum. In particular, the cells are not washed after separation from the trypsinized buffer. The supernatant buffer is decanted and the trypsin that remains with the cells is effectively antagonized by trypsin inhibitor which is included in the incubation medium.

The intensity of mechanical agitation is an important factor in determining the number of cells dispersed and the functional capacity of the isolated cells. In a series of preliminary experiments it was shown that when the stirrer is revolved too slowly (<80 rpm) the cell count is low (<200,000/ml) and steroid production is low. When the stirrer is revolved too rapidly (300 to 400 rpm) the cell count is also low (<150,000). In the latter instance, the tissue rapidly disintegrates, most of the tissue being dispersed in the first two 20-min incubation periods, but many of the isolated cells are damaged and disrupted. Corticosterone production is definitely low when mechanical agitation is too intense.

Three different lots of trypsin were employed and found effective in the present study. In contrast, collagenase preparations vary in their ability to disperse adrenal tissue into cells responsive to ACTH (1). The combination of enzymes (trypsin, DNase, RNase, collagenase and hyaluronidase) as employed by Halkerston and Feinstein (2) may result in fewer clumps of cells as compared to trypsin alone. However, the technic we have described in which trypsin alone is employed has the decided advantage of simplicity and what is most important yields cells which respond with a high degree of uniformity to small quantities of ACTH.

The isolated adrenal cell suspension has obvious potential as a sensitive and accurate method for assay of ACTH. Ten μU of ACTH induced significant increases in corticosterone production in 4 of 5 experiments. The most sensitive *in vivo* bioassay technics

involving hypophysectomized rats, the adrenal vein tap technic of Lipscomb and Nelson (6) and the adrenal corticosterone method of Vernikos-Danellis *et al.* (7) have minimum effective doses of about 50 μU of ACTH. The isolated adrenal suspensions prepared by collagenase as described by Kloppenborg *et al.* (1) exhibited increased corticosterone production in response to 40 μU of ACTH.

Since inter-animal variation is eliminated by combining adrenals from 16 rats to prepare a pool of isolated cells, a relatively high degree of accuracy is predicted. This is borne out by the index of precision (λ) values for experiments included in Table I (see Table III). Values for s and b are included in this table.

Summary. A simple and reproducible technic for the preparation of active isolated rat adrenal cells has been described. Quartered glands are incubated at 37° with mechanical agitation in the presence of trypsin (0.25%). The dispersed cells are collected by centrifugation and suspended in Krebs–Ringer bicarbonate buffer to which has been added glucose (0.2%), albumin (3.0%) and trypsin inhibitor (2 mg/ml). Aliquots of the suspension are incubated at 37° for 2 hr. ACTH in concentrations of 10, 40, and 160 μU increases corticosterone production by the isolated cells. The regression line relating corticosterone production and log dose of ACTH is straight.

TABLE III. Statistical Evaluation.

Expt. no.	*s*	*b*	λ
1	0.275	3.40	0.08
2	0.334	2.09	0.16
3	0.134	2.26	0.06
4	0.148	3.38	0.04
5	0.116	2.12	0.05

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1. Kloppenborg, P. W. C., Island, D. P., Liddle, G. W., Michelakis, A. M., and Nicholson, W. E., Endocrinology **82**, 1053 (1968).

2. Halkerston, I. D. K. and Feinstein, M., Federation Proc. **27**, 626 (1968).

3. Bangham, D. R., Mussett, M. V., and Stack-Dunne, M. P., Bull. World Health Organ. **27**, 395 (1962).

4. Silber, R. H., Busch, R. D., and Oslapas, R., Clin. Chem. **3**, 278 (1963).

5. Baker, F. J., "Handbook of Bacteriological Technic," p. 25. Appleton, New York (1967).

6. Lipscomb, H. S. and Nelson, D. H., Endocrinology **71**, 13 (1962).

7. Vernikos-Danellis, J., Anderson, E., and Trigg, L., Endocrinology **79**, 624 (1966).

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