Guinea Pig Adrenocortical Cells: In Vitro Characterization of Separated Zonal Cell Types

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ABSTRACT This paper reports a quick, relatively simple and reproducible technique for obtaining populations of zona fasciculata and zona glomerulosa cells up to 80–90% pure, which can be maintained in vitro for study of adrenocortical cell function. Isolated guinea pig adrenocortical cells were separated on a 1–28% bovine serum albumin/Ca^{++}, Mg^{++}-free buffer gradient (wt/vol at 4% increments) using equilibrium density centrifugation (570 g, 30 min). Over 60% of the 8×10^5 viable cells/adrenal obtained in the total isolate were recovered after separation. 80% of the zona glomerulosa cells were found in the lower three bands of the gradient. 78% of the zona fasciculata cells were found in the top three bands. Of the cells in the first two bands, 78–91% were zona fasciculata cells, whereas of the cells in the bottom two bands 92–95% were zona glomerulosa cells. The cells retained the morphological characteristics of cells *in situ* and could be maintained in vitro for periods up to 11 d. They produced a wide variety of steroids, cortisol, corticosterone, aldosterone, 11-β-hydroxyandrostenedione, deoxycortisol, deoxycorticosterone, cortisone, 18-hydroxycorticosterone, and a product tentatively identified as dehydroepiandrosterone, and they responded to ACTH in a dose-responsive manner with enhanced levels of steroid output. Zona glomerulosa-enriched populations differed from zona fasciculata-enriched populations in their abundant production of aldosterone and in the pattern of steroid production. None of the cultures responded to angiotensin II (100 pg/ml) with increased steroid production.

Analysis of the functions of adrenocortical cells has been facilitated by utilization in vitro of populations of cells enriched for the various cell types (4, 5, 38, 40). Of the available techniques, however, those which effectively separate cell populations are time-consuming, and those that are shorter sacrifice the purity of the populations obtained. Most such studies have employed freshly isolated cells, which may have been altered by the isolation procedure and may still be influenced by prior exposure to hormones in vivo. Few studies have examined cell function after longer periods in vitro.

Part of the problem in separating the cells is the similarity, in most species, of their size and density, as well as their morphological features. The guinea pig adrenal, on the other hand, has a cortex with four morphologically distinct zones[1] and clearly distinguishable cell types (8). It secretes cortisol, corticosterone, aldosterone, cortisone, 11-deoxycortisol (14), 11-β-hydroxyandrostenedione, and androstenedione (13). In addition, it is capable of synthesizing conjugated steroids such as dehydroepiandrosterone sulfate (24, 25, 39). There is also

some evidence that the distinct zonal cell types may have distinct functional properties. The zona reticularis has been shown to contain the enzyme involved in the sulfation of dehydroepiandrosterone (24), and the zona fasciculata and zona reticularis cells respond differently to ACTH and dexamethasone treatment (9).

Taking advantage of their differences in size and density, we have been able to efficiently separate guinea pig zona glomerulosa from zona fasciculata cells on a bovine serum albumin (BSA)/Ca^{++}, Mg^{++}-free step gradient. The entire procedure is simple and reproducible, and can be carried out by one individual using easily obtainable materials and equipment. The cells were maintained for up to 11 d in culture, allowing sufficient time for them to recover from any trauma induced by the isolation and separation procedure, as well as to adapt to culture conditions and be cleared of in vivo hormonal effects before being examined for their ability to respond to ACTH and angiotensin II. This adrenocortical cell system offers one the additional advantage of performing successive experiments

on the same cell populations and thus gaining insight into a dynamic continuum of cell function not unlike that which would occur in vivo.

MATERIALS AND METHODS

Isolation and Separation of Guinea Pig Adrenocortical Cells

Adrenals from six adult male English short-hair guinea pigs (Camm Research Lab Animals, Wayne, NJ) were used in each cell isolation. Animals were anesthetized with Diabutal, and their adrenals were removed aseptically and placed immediately in ice-cold Krebs-Ringer's bicarbonate buffer (KRB). The adrenals were trimmed of adherent fat, rinsed in KRB, and weighed. Cells were isolated using an adaptation[1] of the procedure of Hopkins and Farquhar (20), which employs incubation in trypsin and collagenase followed by incubation and mechanical shearing in KRB lacking Ca^{++} and Mg^{++} (IF-KRB). All solutions were equilibrated with 95% O_2 and 5% CO_2 to pH 7.4 and Millipore-filtered (0.45 μM; Millipore Corp., Bedford, MA).

The pellet of isolated cells (total isolate) was resuspended in 1% BSA/IF-KRB (BSA, fraction V; Sigma Chemical Co., St. Louis, MO) and loaded onto the top of a 1-28% BSA/IF-KRB (wt/vol) step gradient (4 ml/step and at 4% increments after the first 1% step). The BSA solutions were prepared the day before, Millipore-filtered, and kept at 4°C. The densities of the BSA solutions at 4°C were determined by refractometry (Bausch and Lomb refractometer; Bausch & Lomb, Inc, Rochester, NY) and by using the formula: density = −0.925 + (1.45) refractive index (7). The gradient was spun in a 50-ml siliconized Kimax glass centrifuge tube at 4°C in an International PR-2 centrifuge (International Equipment Co., Needham Heights, MA) at 570 g for 30 min. Six bands of cells, at the interfaces of 4/8, 8/12, 12/16, 16/20, 20/24, and 24/28% BSA/IF-KRB, plus a pellet of erythrocytes in 28% BSA, were collected from the top of the gradient with sterile Pasteur pipettes in a 4°C cold room. The cells in each band were washed with KRB and pelleted by centrifugation at 200 g for 5 min. Cells were then suspended in culture medium (MEM; Grand Island Biological Co., Grand Island, NY [Gibco]; supplemented as described below). Aliquots of the total isolate and of each band off the gradient were stained with 0.4% trypan blue and cells counted in a hemocytometer to determine the yield of viable cells and the relative proportion of each zonal cell type. Zona fasciculata externa and interna cells were counted together as zona fasciculata. Light micrographs of the cells were photographed using a Wild (Wild Heerbrugg Instruments Inc., Farmingdale, NY) inverted photomicroscope.

Cell Culture

Enriched populations of guinea pig zona glomerulosa and zona fasciculata cells obtained after separation by equilibrium density centrifugation were plated in 35 mm plastic culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) at 1.5×10^5 cells/ml/dish or in 15 mm multiwells (Falcons Labware) at 0.75×10^5 cells/0.5 ml/well. MEM was supplemented with 12.7% (vol/vol) fetal calf serum (Gibco), 0.29 mg/ml glutamine (Gibco), 50 μg/ml vitamin C (Gibco), 1% each of MEM vitamins and nonessential amino acids, 4 μg/ml Fungizone (E. R. Squibb & Sons, Princeton, NJ) and 2 μg/ml Gentamicin (Schering, Port Reading, NJ). Cultures were maintained in 5% CO_2 in a humidified incubator (Forma Scientific, Marietta, OH).

EFFECTS OF ACTH: Cultures were treated with or without ACTH (Acthar, Armour Pharmaceutical Co., Phoenix, AR) at: (a) 0.1-100 mU/ml for 48 h on day 2 of culture; (b) 100 mU/ml for 48 h periods on days 7 and 9 of culture; (c) 100 mU/ml for 2 h (acute) at 6 h or 12 h after plating, followed by daily 2 h treatments for 6 days.

EFFECTS OF ANGIOTENSIN II: Cultures were treated with 100 pg/ml of angiotensin II (Bachem, Torrance, CA) for periods of 2, 4, 12, or 24 h on day 3 of culture.

Electron Microscopy

Isolated cells and bands of separated cells were pelleted and fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min and processed for electron microscopy[1]. Cultures enriched in zona glomerulosa and zona fasciculata cells were fixed at different time intervals and processed in situ as done previously[1]. Sections, stained with uranyl acetate and lead citrate, were examined in a Siemens IA electron microscope.

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[1] Black, V. H., J. Mierlak, T. Katz, P. Miao, T. Huima, and N. McNamara. Isolated guinea pig adrenocortical cells in vitro: morphology and steroidogenesis in control and ACTH-treated cultures. Manuscript submitted for publication.

Steroid Assay Procedures

Media collected from all cultures (1 ml or 0.5 ml in volume) were spun at 200 g for 5 min to pellet debris. Supernatants were stored at −70°C until assayed. A 0.1-ml aliquot of the supernatant was treated with 10 μl of 0.25 N NaOH and steroids were extracted with 4 ml of methylene chloride (29).

THIN LAYER CHROMATOGRAPHY: Methylene chloride extracts of media from similar cultures from the same time period and treatment were pooled and dried under nitrogen. The dried extracts were chromatographed on precoated silica gel G60 plates (20 × 20 cm, E. Merck, Darmstadt, Germany). Chromatography was carried out in three different systems: methylene chloride:methanol:water (150:9:9:0.5), benzene:acetone:water (75:50:0.2), and chloroform:methanol (95:6). Plates were air-dried and treated with 2% H_2SO_4 in ethanol and then heated at 110°C for 20 min. Fluorescent spots were visualized with ultraviolet (UV) light at 256 nm. Cortisol (hydrocortisone; Sigma Chemical Co.), corticosterone (Calbiochem-Behring Corp., San Diego, CA), and aldosterone (d-aldosterone; Sigma Chemical Co.) were used as standards on each plate.

SPECTROFLUOROMETRIC ASSAY: The method of Mejer and Blanchard (29) was used to quantitate the fluorogenic steroids, principally cortisol and corticosterone. Methylene chloride extracts were reacted with the fluorescence-inducing reagent (H_2SO_4:ethanol, 65:35, vol/vol). Fluorescence was read at 520 nm with an excitation wavelength of 470 nm, using an Aminco Bowman spectrophotofluorometer (American Manufacturing Co., Inc., Silver Springs MD).

Fluorescence units were expressed as micrograms of cortisol per milliliter from the standard curve run with each assay (21). In addition, because cortisol produces only 33% of the fluorescence of corticosterone (11, 32; Martin and Black, unpublished results), the fluorescent intensity (units) of each sample, although expressed as microgram equivalents of cortisol, represents the sum total of steroid fluorescence, and the absolute levels of neither cortisol or corticosterone. The fluorescent intensity of medium controls (medium incubated in culture dishes without any cells) was similar to that of the blank. In cases where it was higher than the blank it was subtracted from the sample intensities.

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC): Methylene chloride extracts of culture media were dried under nitrogen and analyzed by reverse phase high pressure liquid chromatography as described by O'Hare et al. (31). They were redissolved in dioxane and injected into a Spectra-Physics SP 8000 microprocessor-controlled HPLC (Spectra-Physics Inc., Mountain View, CA) and separated on a Zorbax-C8 (5 μm) column (100 × 5 mm, inner diameter) using a 40-100% nonlinear gradient of methanol-water. All chromatograms were obtained at 45°C, and eluted steroids were detected at 240 nm using a Schoeffel 770 variable wavelength spectrophotometer (Schoeffel Instruments, Div. Kratos, Inc., Westwood, NJ). Non-UV-absorbing steroids were detected by radioscans of culture media treated with [7-^{3}H]pregnenolone (New England Nuclear, Boston, MA; 17.2 Ci/mmol, 1 μCi/ml). Fractions were collected from the chromatograph and radioactivity quantitated by liquid scintillation counting. All HPLC was performed by Dr. M. O'Hare, Ludwig Institute for Cancer Research, Royal Marsden, England.

RADIOIMMUNOASSAY OF ALDOSTERONE: Aldosterone present in the culture medium was quantitated by radioimmunoassay using the method of Mayes et al. (27) with some modifications. Aliquots of media were spiked with 3000 cpm of ^{3}H-aldosterone (D-[1,2,6,7-^{3}H (N)]-aldosterone, 80–105 Ci/mmol in benzene-ethanol, New England Nuclear) before methylene chloride extraction. The aqueous phase was separated by phase separating paper (IPS, Whatman Inc., Chemical Separation Div., Clifton, NJ) and discarded. The methylene chloride extracts were chromatographed (descending) overnight on 3 MM paper (Whatman Inc., Paper Div., Clifton, NJ) using the upper phase of the chromatographic solution (water:toluene:methanol:hexanes, 2:18:10:5). Sample aldosterone was located by interpolation of distances traveled by ^{3}H-aldosterone standards as determined by strip scanning (Actigraph III, Nuclear Chicago Corp., Des Plaines, Il). The samples were eluted with redistilled methanol and assayed in 10 × 75 mm borosilicate culture tubes (Fisher Scientific Co., Pittsburgh, PA). The antiserum was raised in rabbits immunized with BSA-conjugated 3-oxime-aldosterone. The final antibody solution (1:2,500) was prepared in 0.05 M borate buffer (pH 8, 2% methanol) containing 0.5% gamma globulin. After overnight incubation in the refrigerator, bound antibody was precipitated with a saturated ammonium sulfate solution. The amount of aldosterone was determined from percent free counts in the supernatant, using a standard curve run with each assay.

RESULTS

Yield and Recovery

Isolation of the adrenocortical cells was completed within 2–3 h after removal of the glands. Microscopic examination of all supernatant solutions in which adrenal fragments had been incubated or suspended before mechanical dissociation showed absence of viable adrenocortical cells. The total isolate con-

tained zona glomerulosa, zona fasciculata externa, zona fasciculata interna, and zona reticularis cells, as well as erythrocytes, connective tissue elements, and cell debris (Figs. 2, 3, 8). The yield and recovery of cells in the total isolate is summarized in Table I and is in good agreement with that found previously.[1]
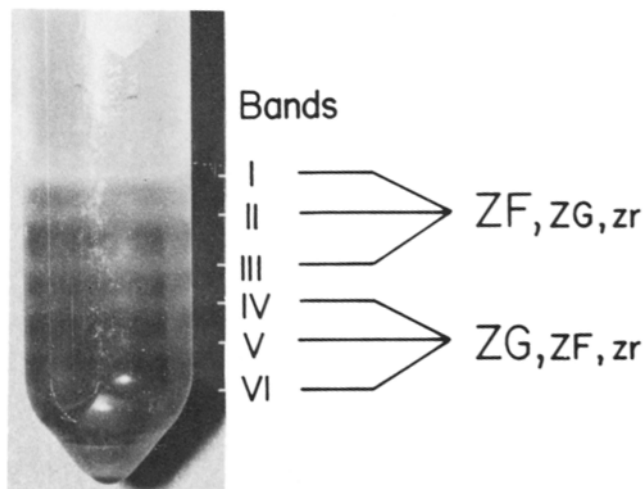

FIGURE 1 BSA step gradient (4–28%) immediately after equilibrium density centrifugation. The cells are loaded onto the gradient in 1% βSA and separate during centrifugation (570 g, 30 min) into discrete bands at the interfaces of the gradients increment steps (4% each). Bands I–III are predominantly zona fasciculata (ZF) cells, with some zona glomerulosa (ZG) cells and very few zona reticularis (ZR) cells. Bands IV–VI are predominantly ZG cells, some ZF and very few ZR cells. A pellet consisting mostly of erythrocytes is visible at the tip of the gradient tube. The densities (g/cm³, at 4°C) of the BSA solutions are as follows: 1%, 1.0131; 4%, 1.0209; 8%, 1.0305; 12%, 1.0393; 16%, 1.0486; 20%, 1.0583; 24%, 1.0682; 28%, 1.0782.

Six bands and a pellet of cells were collected from the BSA step gradient after equilibrium centrifugation of the total isolate (Fig. 1). The yield of viable cells after separation was ~62 × 10⁵ cells (Table I). This represented over a 60% recovery of all adrenocortical cells present in the total isolate, with no preferential loss of any specific cell type (68% of the zona glomerulosa cells, 58% of the zona fasciculata cells, and 66% of the zona reticularis cells).

Of all zona fasciculata cells, 78% banded at densities 1.02–1.05 g/cm³ (bands I–III) while 80% of the zona glomerulosa cells banded at densities 1.05–1.07 g/cm³ (IV–VI). Most of the zona reticularis cells banded at densities 1.03–1.05 g/cm³ (III–IV), intermediate between zona fasciculata and zona glomerulosa cells (Table II).

Pooling cells from bands I–III of the step gradient after separation provided us with a population of ~80% zona fasciculata cells and ~20% zona glomerulosa cells. Pooling bands IV–VI gave us the reverse distribution. However, since the final yield of zona glomerulosa cells is high compared with zona fasciculata cells, bands IV, V, and VI frequently were not pooled before they were plated. These individual bands were up to 95% pure (band VI) in zona glomerulosa cells. Although ~70% of the zona reticularis cells were found in bands III–V, these cells comprised only 5% of the cells in these bands. This is due, at least in part, to the low yield of zona reticularis cells in the total isolate. For although 66% of the zona reticularis cells were recovered after separation, they represented only 3.5% of the viable cells in the total isolate.

Morphology and Fine Structure of Isolated and Separated Cells

To monitor morphological integrity, cells were examined at both light (phase contrast) and electron microscopic levels at

TABLE I

Quantitative Analysis of Cells after Isolation and Separation on a BSA Step Gradient

	Total	ZG	ZF	ZR
Total cells obtained				
Number of viable cells (× 10⁵) in total isolate	96.04 ± 8.92	65.49 ± 6.32	27.23 ± 2.76	3.32 ± 0.56
Number of viable cells (× 10⁵) after separation	62.24 ± 7.65	44.33 ± 5.63	15.73 ± 2.28	2.18 ± 0.46
Recovery per adrenal				
Number of cells (× 10⁵) per adrenal*	120.41	30.54	46.67	43.20
Number of viable cells (× 10⁵) in total isolate per adrenal	8.00	5.45	2.10	0.46
Number of viable cells (× 10⁵) per adrenal recovered after separation	5.19	3.69	2.10	0.18
Percentage of viable cells per adrenal recovered after isolation	6.67	17.84	4.50	1.06
Percentage of viable cells per adrenal recovered after separation	4.31	12.1	2.81	0.42

The number of cells per adrenal was calculated as outlined in Black et al. (See footnote 1) and is presented for animals of 750–800 g body weight. The rest of the data represents the average of 12 experiments, using six guinea pigs, each weighing ~800 g, per experiment.
* See footnote 1.

TABLE II

Quantitative Analysis of the Distribution of ZG, ZF (Externa Plus Interna), and ZR Cells in Each Band of the Gradient after Separation

	Percentage of total cells recovered	ZG		ZF		ZR	
		Percentage of total (yield)	Percentage of band (purity)	Percentage of total (yield)	Percentage of band (purity)	Percentage of total (yield)	Percentage of band (purity)
Band I	1.1 ± 0.2	0.2 ± 0.1	7.9 ± 2.2	4.0 ± 0.6	91.5 ± 2.4	0.1 ± 0.1	0.6 ± 0.5
Band II	11.9 ± 1.9	3.9 ± 0.8	20.2 ± 2.3	36.0 ± 4.2	78.4 ± 2.6	3.2 ± 1.2	1.4 ± 0.6
Band III	22.5 ± 3.0	15.0 ± 2.9	44.1 ± 4.8	38.2 ± 3.1	50.5 ± 5.2	29.6 ± 5.5	5.5 ± 1.9
Band IV	26.3 ± 2.0	29.2 ± 2.5	77.7 ± 3.4	16.2 ± 2.4	17.2 ± 2.9	39.0 ± 4.0	5.0 ± 0.9
Band V	23.9 ± 2.9	31.7 ± 3.3	92.2 ± 2.0	3.7 ± 0.7	4.8 ± 1.6	18.6 ± 3.6	2.9 ± 0.8
Band VI	14.5 ± 2.4	20.0 ± 3.1	95.1 ± 0.8	2.2 ± 1.1	3.1 ± 2.8	9.1 ± 2.8	1.8 ± 0.5

Yield was obtained by: number of cells (type) in band/total number of cells (type) recovered after separation × 100%. Purity was obtained by: number of cells (type) in band/total number of all cell types in same band × 100%.

points throughout the isolation and separation procedures. Intact cells of all four cortical cell types (8) were easily identifiable (Figs. 2-11). Zona glomerulosa cells could be distinguished from other cells by their smaller size (13 μm in diameter), few lipid droplets, and intended nucleus (Figs. 2, 3, 7, 11). Zona fasciculata externa and zona fasciculata interna cells were intermediate in size (22-30 μm in diameter) and were characterized by abundant lipid droplets (Figs. 2-5, 9). These
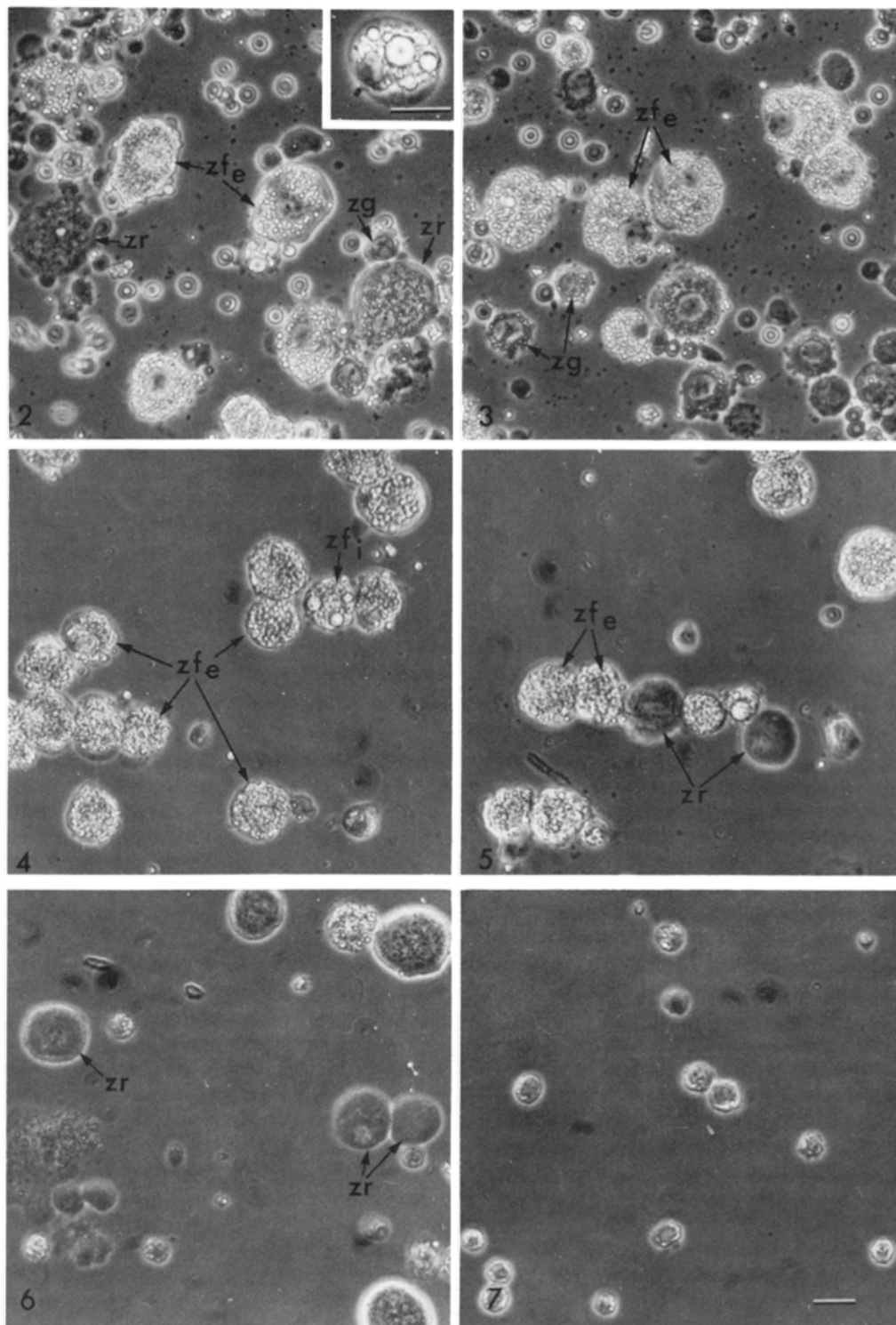
FIGURES 2 and 3   Phase-contrast micrographs of a total isolate immediately after cell isolation. It contains zona glomerulosa (zg), zona fasciculata externa (zfₑ), zona fasciculata interna (inset, × 440) and zona reticularis (zr) cells. Bar, 20 μm. × 300.

FIGURES 4-7   Phase-contrast micrographs of cells immediately after cell separation. Band II (Fig. 4) contains predominantly zona fasciculata externa (zfₑ) and zona fasciculata interna (zfᵢ) cells. Band III (Fig. 5) contains mainly zona fasciculata externa (zfₑ) and some zona reticularis (zr) cells. Band IV (Fig. 6) contains zona reticularis (zr) cells as well as zona glomerulosa cells. Band VI (Fig. 7) contains predominantly zona glomerulosa cells. Bar, 20 μm. × 300.
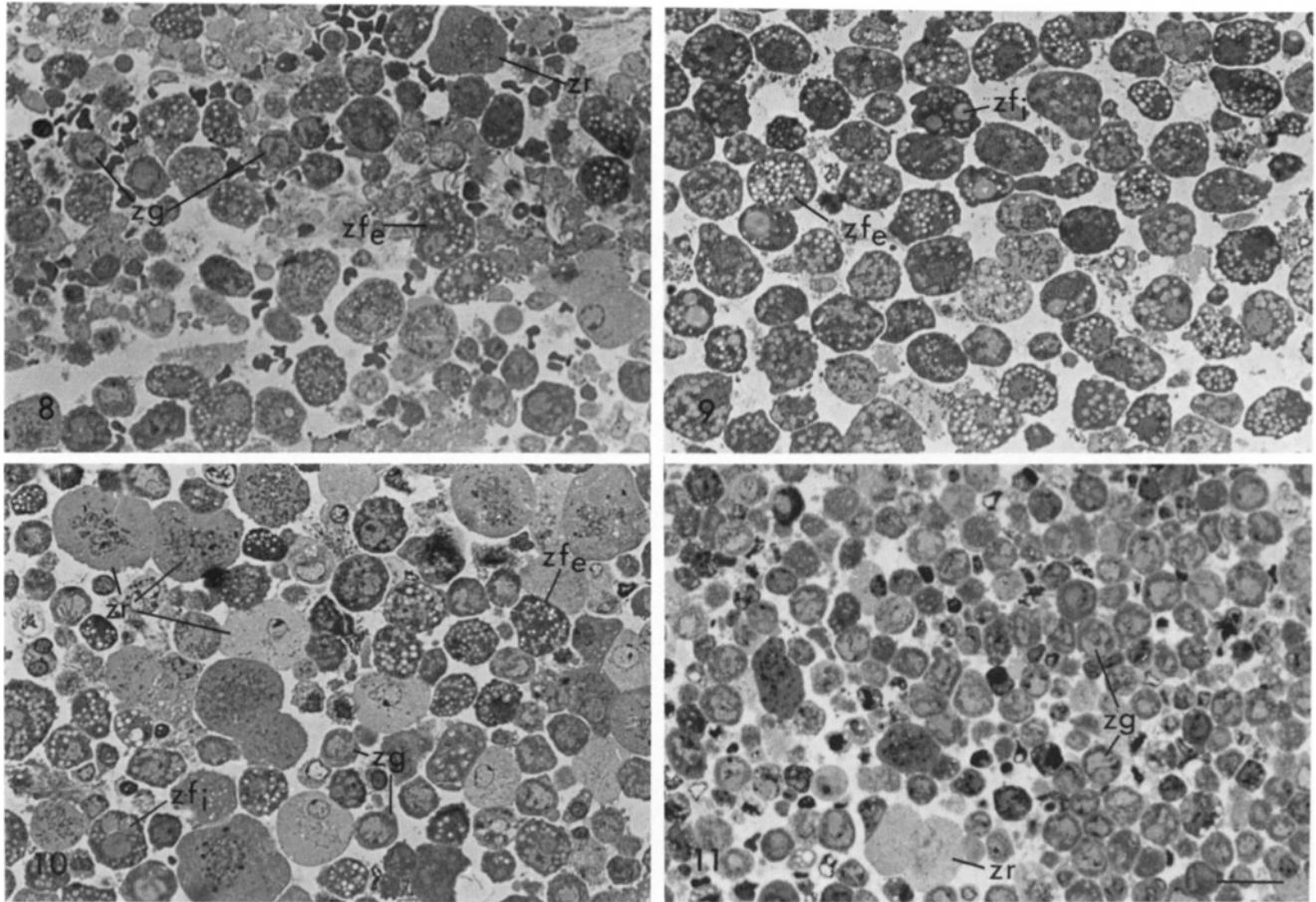
FIGURE 8 Light micrograph of a 1.5-µm section of an Epon-embedded pellet of the total isolate. It contains zona glomerulosa (zg), zona fasciculata externa (zf$_e$), zona fasciculata interna (not shown), and zona reticularis (zr) cells as well as erythrocytes, connective tissue elements, and cell debris. Bar, 20 µm. × 140.

FIGURES 9–11 Light micrographs of 1.5-µm sections of Epon-embedded pellets of bands from the BSA gradient. Band III (Fig. 9) contains predominantly zona fasciculata externa (zf$_e$) and interna (zf$_i$) cells. Band V (Fig. 10) contains predominantly zona glomerulosa (zg) cells and some zona reticularis (zr) and zona fasciculata externa (zf$_e$) cells. Band VI (Fig. 11) contains predominantly zona glomerulosa (zg) cells and a few zona reticularis (zr) cells. Bar, 20 µm. × 140.

two cell types could be distinguished as in situ (8, 9) by the heterogeneity of lipid droplet size in the zona fasciculata interna cells (Figs. 2, 4, 9). The zona reticularis cells were largest (~28 µm in diameter) and had a characteristically clear rim of peripheral cytoplasm free of granules and lipid droplets (Figs. 5, 7, 10). The cells retained most of the fine structural features previously described as characteristic of each cell type in situ (8, 9). However, mitochondria of all the isolated cortical cells were more electron dense than in situ and some vesicularization of smooth endoplasmic reticulum occurred.

Morphology and Fine Structure of Cultured Cells

To examine the behavior of the separated cells after plating, cultures were examined by phase contrast and electron microscopy at different time intervals (Figs. 12–18). The attached cells recovered from the changes in mitochondria and smooth endoplasmic reticulum which took place during isolation and separation (Figs. 16–18) and retained a good morphological appearance throughout the 11-d culture period. However, with time and after ACTH treatment, it became increasingly difficult in the mixed culture populations to distinguish zona glomerulosa from zona fasciculata cells on the basis of their

fine structure. A similar observation in cultures of total isolates had led us to propose that changes occurred in mitochondria and smooth endoplasmic reticulum in the zona glomerulosa cells.[1] Examination of the enriched populations of zona glomerulosa cells confirmed that this did occur. Some of the cells developed enlarged pleiomorphic mitochondria with tubular cristae and a greater amount of smooth endoplasmic reticulum. These changes were enhanced after ACTH treatment. Changes in zona fasciculata cells were also better defined in their enriched cell populations. These cells showed a marked increase in smooth endoplasmic reticulum and a decrease in lipid droplet size, similar to changes previously quantitated for the cells in situ (9).

Functional Characteristics of Cultures Enriched with Zona Fasciculata and Zona Glomerulosa Cells

To examine the functional capabilities of separated guinea pig adrenocortical cells in vitro, cultures enriched in zona fasciculata or zona glomerulosa cells and cultures with mixed cell types were maintained for different periods (below) in the presence or absence of ACTH and angiotensin II.

The dose-response of cells to ACTH was examined on day
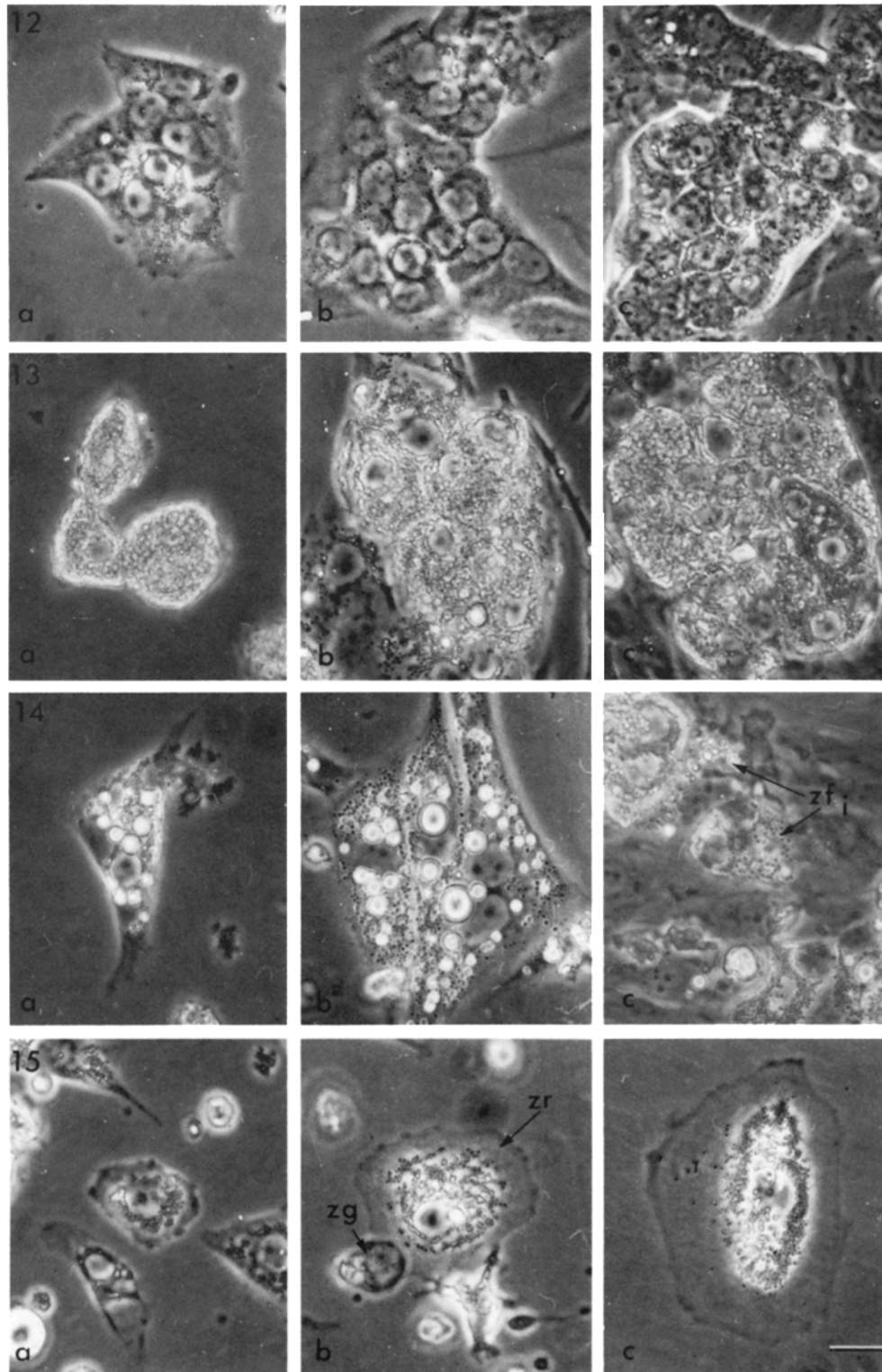
FIGURES 12–15 Phase-contrast light micrographs of cells cultured for (a) 1, (b) 3, and (c) 7 d after separation. Zona glomerulosa cells from band VI (Fig. 12). Zona fasciculata externa cells from band II (majority in Fig. 13). Zona fasciculata interna cells from band II (Fig. 14). Zona reticularis cells from band V (Fig. 15). Bar, 20 μm. × 180.

2. Cultures enriched in both zona glomerulosa and zona fasciculata cells responded to increasing levels of ACTH with increased fluorogenic steroid production (Fig. 19). Similar results were obtained at later time points, although the maximal levels achieved after day 7 were lower (data not shown).

Thin layer chromatography (data not illustrated) indicated that both cortisol and corticosterone were produced at basal levels and increased in response to ACTH. Aldosterone was not consistently detectable in thin layer chromatograms, but was observed in media from some populations highly enriched with zona glomerulosa cells (bands V and VI), and increased after ACTH treatment.

To assess the pattern of steroid output over the 11-d period, culture media were assayed for fluorogenic steroids (cortisol
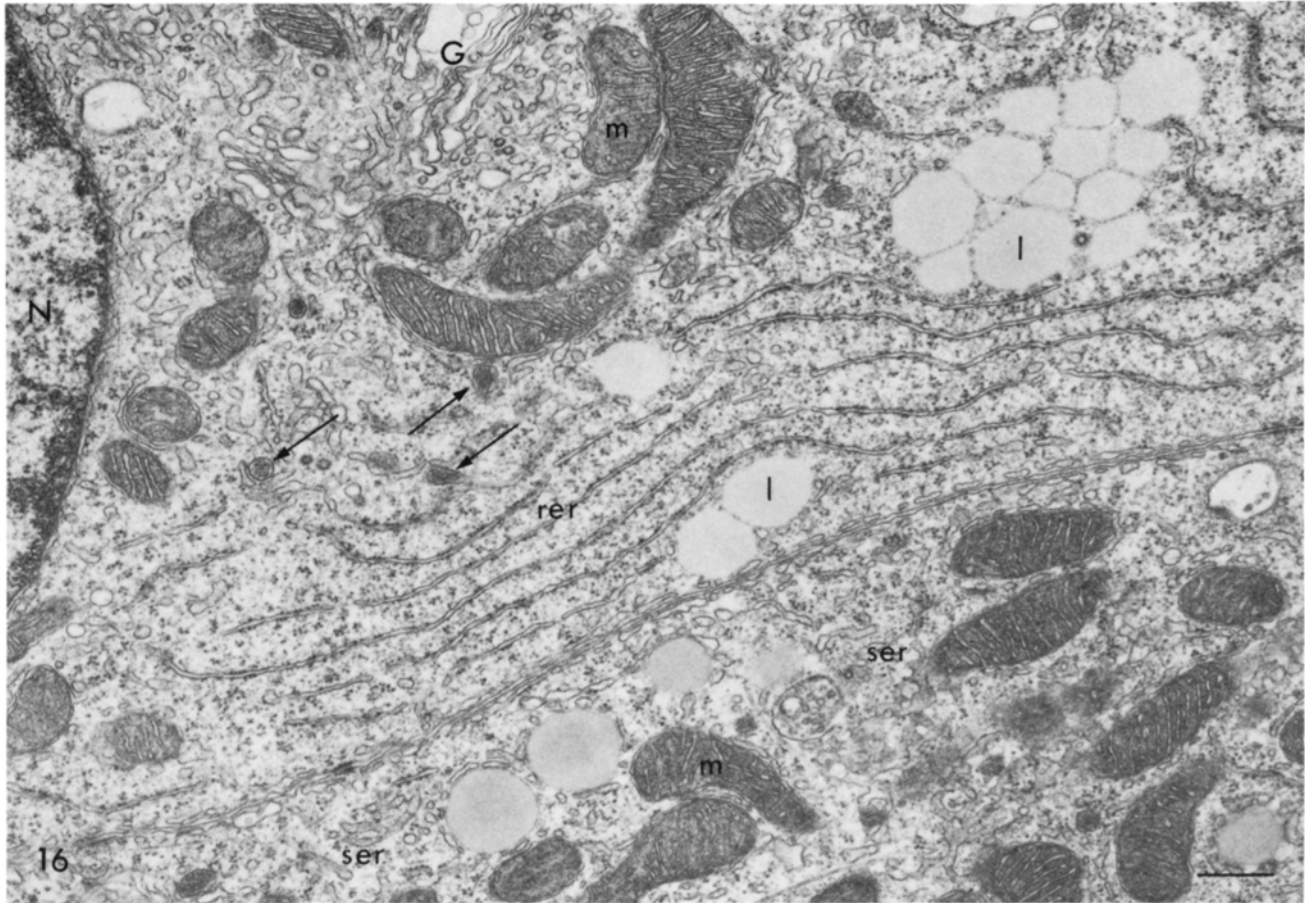
FIGURE 16 Electron micrograph of portions of two zona glomerulosa cells cultivated 2 d in vitro. These cells have long cisternae of rough endoplasmic reticulum (*rer*) and some tubular smooth endoplasmic reticulum (*ser*). The mitochondria (*m*) are elongated with moderately dense matrices. Elements of the prominent Golgi complex (*G*) are seen adjacent to the nucleus (*N*). Peroxisomes (arrows) are scattered throughout the cytoplasm. Lipid droplets (*l*) occur in small groups. Bar, 0.5 μm. × 20,500.

and corticosterone) and aldosterone. Steroid production, measured as 48-h accumulations, increased with time in all cultures, with fluorogenic steroids peaking at day 7 (Fig. 20) and aldosterone peaking at day 9 (Fig. 21). When treated with ACTH at 7 and 9 d, the ratio of ACTH to control levels of fluorogenic steroid production was highest in zona fasciculata-rich cultures and these cultures showed an increased response to ACTH during their second 48-h period of treatment (Fig. 20). Zona glomerulosa-rich cultures did not show such an increased response (Fig. 20). Aldosterone reached the highest absolute levels in response to ACTH in zona glomerulosa-rich cultures but mixed cultures showed the greatest response in terms of the ratio of ACTH to control levels (Fig. 21). Although the levels of aldosterone dropped from day 9 to 11, the ratios of ACTH to control levels remained similar (Fig. 21).

When measured daily for a period of 2 h over 6 d (Table III), the basal level of the fluorogenic steroids and that induced by ACTH remained fairly constant in zona fasciculata-enriched cultures. However, the basal level in zona glomerulosa-enriched populations dropped significantly after the first day. Only after day 1 was a significant ACTH response discernible, but it then remained fairly constant for the remainder of the culture period. Analysis of steroid levels accumulated in ACTH-free media after the 2-h treatment indicated that the ACTH response continued in the absence of ACTH (Table III).

HPLC analysis of media from 1-wk-old cultures confirmed

and extended the above results (Fig. 22). Cortisol and corticosterone were produced by all cultures. These two steroids comprised a greater percentage of the total steroid output in zona fasciculata-rich cultures, but both the absolute amounts and the ratio of cortisol to corticosterone were higher in the zona glomerulosa-rich cultures. Aldosterone was barely detectable in zona fasciculata-rich cultures, but comprised increasingly greater percentages of total steroid output in populations with increasing percentages of zona glomerulosa cells. The ratio of aldosterone to corticosterone was also higher in zona glomerulosa-enriched cultures. In addition, all cultures were shown to produce 11-β-hydroxyandrostenedione, with zona glomerulosa-enriched cultures producing more than zona fasciculata-enriched cultures, while cortisone, deoxycortisol, deoxycorticosterone, and 18-hydroxycorticosterone were detectable only in mixed and zona glomerulosa-rich cultures. A non-UV-absorbing peak, detected in the radioscans, in a position consistent with its being dehydroepiandrosterone or 17-α-hydroxypregnenolone, was seen in all cultures.

HPLC analysis of control and ACTH-treated cultures on day 9 showed that all steroids increased with ACTH, but in differing ratios, depending on the cell population (Fig. 22). Although the absolute levels of cortisol and corticosterone, and the ratio of cortisol to corticosterone were higher in zona glomerulosa-rich cultures, the increase in the ratio of cortisol to corticosterone was greater in zona fasciculata-rich cultures than in zona glomerulosa-rich cultures. In zona glomerulosa-
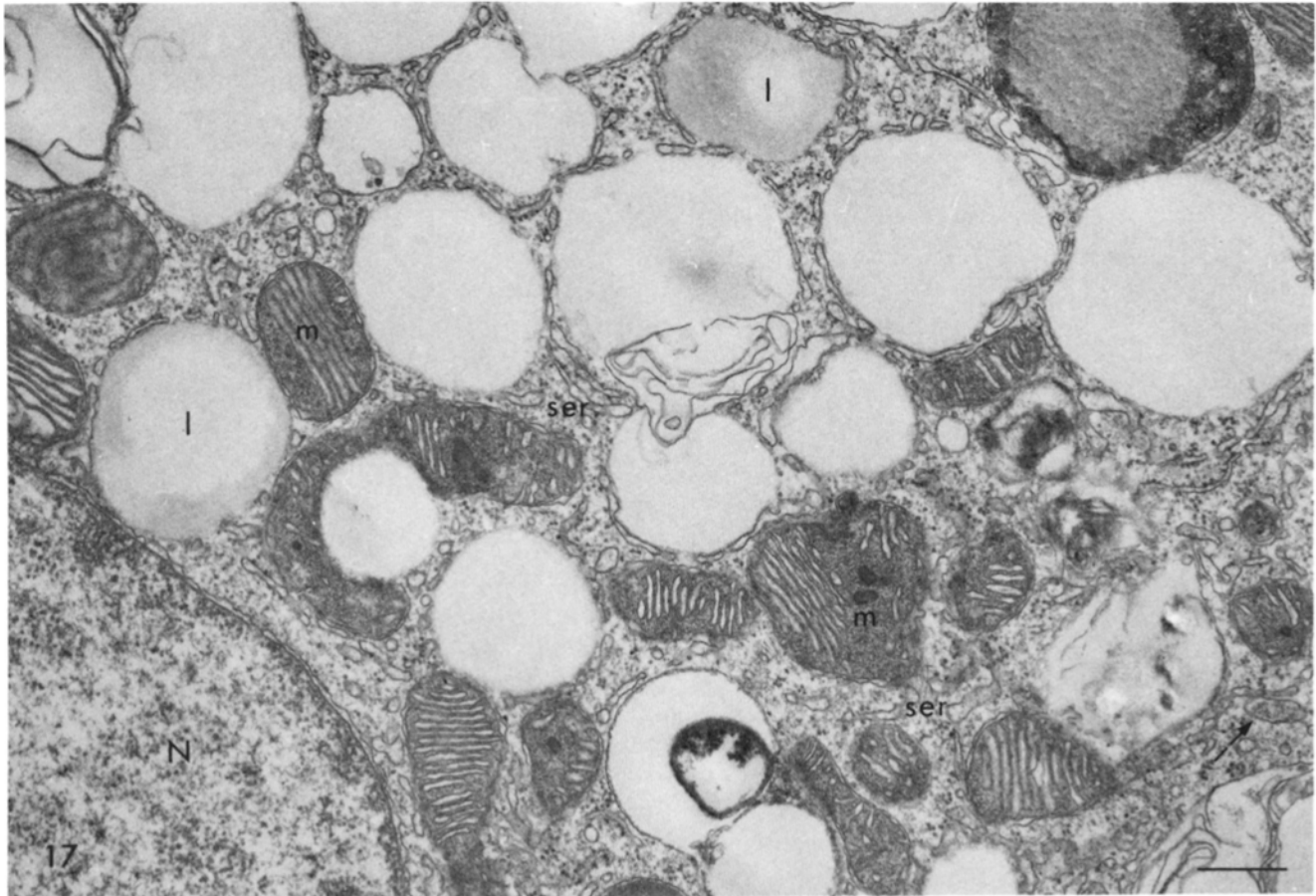
FIGURE 17   Electron micrograph of a portion of a zona fasciculata cell cultivated 2 d in vitro. The mitochondrial matrices (*m*) are less dense in appearance than those of freshly isolated and separated cells. The smooth endoplasmic reticulum (*ser*) is predominantly tubular, with some fenestrated elements occuring at the periphery of lipid droplets (*l*). Peroxisomes (arrows) are scattered throughout the cytoplasm. A portion of the nucleus (*N*) is shown. Bar, 0.5 μm. × 24,000.

enriched cultures, aldosterone secretion was increased, but so was the percentage of total output of cortisol plus corticosterone.

When day 3 cultures were exposed to angiotensin II for periods up to 24 h no difference was seen in aldosterone or fluorogenic steroid production between control and treated cultures (data not shown).

DISCUSSION

Cell Isolation and Separation

In vitro studies have contributed a significant amount of information on the functional diversity and capability of the adrenal cortex, for such analysis can be performed under more defined and controlled conditions than can be achieved in vivo (12, 21–23, 35, 36). However, functional characteristics of isolated cells in suspension or in culture, which are derived from heterogeneous populations composed of different zonal cell types, can provide limited information with respect to zonal specificity. Specificity can only be studied by obtaining more homogeneous populations, that is, populations enriched with a specific cell type. Many different approaches have been used in attempting to obtain specific zonal tissues for study of their functional characteristics. In glands where there are macroscopic differences, microdissection or separation of regions in which specific cell types predominate has been used (18, 33, 34). Where this is not possible, or in combination with micro-

dissection or enucleation, isolation of the cells followed by their separation on gradients of various types has been utilized (1, 4, 5, 10, 28, 35, 36). However, some of these techniques are time consuming (4, 5, 10, 35, 36), while those which are shorter may sacrifice yield and viability of some cell populations (28).

The technique of separation on a discontinuous BSA gradient used in this study can be exactly reproduced and separates bands of cells discretely. It is relatively quick and simple compared with the unit gravity sedimentation procedure. Gradients can be prepared shortly before the completion of cell isolation, and, after centrifugation, because of the stability of the gradient at 4°C, cells in each visible band can be collected with minimal physical disturbance of cells in successive bands. The proportions of cell types in each band can be assessed quickly with the light microscope and do not require sophisticated cell sorter analyses. The degree of enrichment (relative percent in total isolate vs relative percent in each band) obtained in this study compares favorably with that obtained in separations of rat adrenocortical cells by unit gravity sedimentation (35) and column filtration (28). The recovery and viability of cells obtained after separation on the BSA gradient also compare favorably with data from these studies.

Separated cells in culture were shown by thin layer chromatography, fluorometric analysis, radioimmunoassay, and HPLC to be producing corticosteroids and to respond to ACTH treatment with enhanced steroidogenesis. Qualitatively, the array of steroids secreted by the separated zona fasciculata and
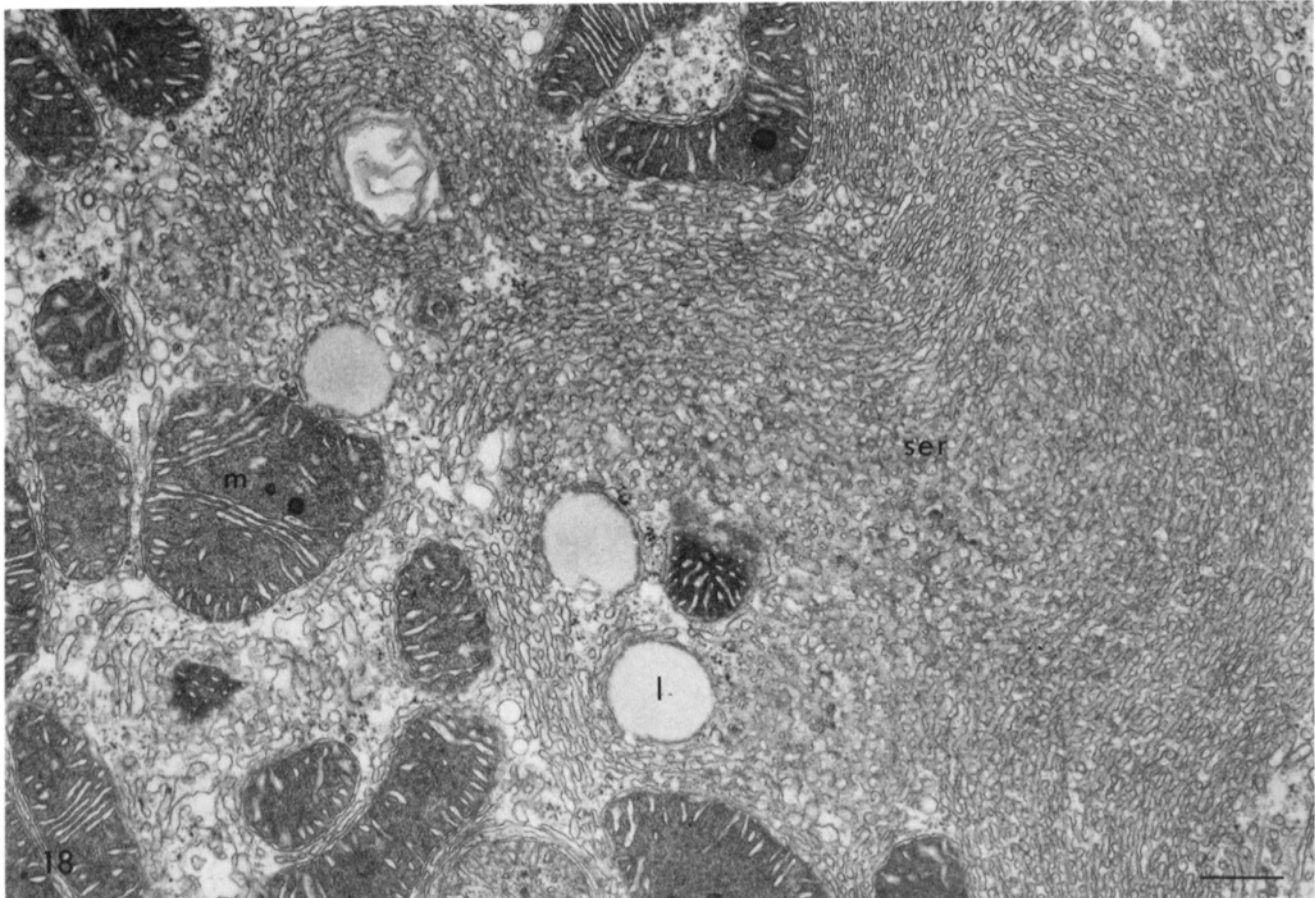
FIGURE 18 Electron micrograph of a portion of a zona reticularis cell cultivated 2 d in vitro. The abundant smooth endoplasmic reticulum (ser) in the peripheral cytoplasm (right half of micrograph) occurs as fenestrated cisternae. Tubular smooth reticulum is in the juxtanuclear area (left half of micrograph) among the other organelles, such as mitochondria (m) and peroxisomes (not shown). Pleiomorphic mitochondria (m) have moderately dense matrices. A few lipid droplets (l) are seen. Bar, 0.5 μm. × 22,000.
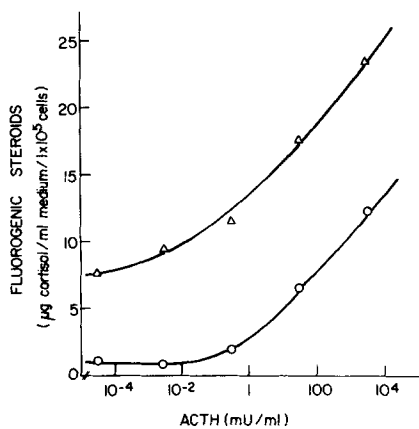

FIGURE 19 Fluorogenic steroid levels obtained in response to increasing levels of ACTH. Cultures were treated for 48 h, beginning on day 2 of culture, with ACTH (10^{-4}-10^4 mU). Zona glomerulosa cell (ZG) enriched populations began at a higher initial level of steroid production but the increase in production over the dose range of ACTH utilized was less than that for zona fasciculata cell (ZF) enriched populations. The points plotted on this graph represent the ACTH-stimulated levels minus the basal level of secretion (3.9 and 9.6 μg of cortisol/ml of medium/1 × 10^5 cells in ZF- and ZG-enriched cell populations, respectively). △, 70% ZG cells; ○, 75% ZF cells.

zona glomerulosa cells is similar to those produced by the guinea pig in vivo (13, 14). Thus, the steroidogenic capability and responsiveness to ACTH of these cells were maintained after cell separation. More significantly, this study, utilizing enriched cell populations, demonstrated that the pattern of basal secretion and the response to ACTH were different in the zona glomerulosa and zona fasciculata. Media from zona glomerulosa–rich cultures had higher levels of fluorogenic steroids than those from zona fasciculata–rich cultures. HPLC analysis indicated that this was due to greater production of corticosterone and cortisol, as well as a variety of other UV-absorbing steroids, including 11-β-hydroxyandrostenedione. This would seem to indicate that, at least after 1 wk in culture, the zona glomerulosa cells have a greater capacity for steroid synthesis. Although we cannot exclude the possibility that this may reflect a differentiation of the zona glomerulosa cells in vitro into cells with zona fasciculata-like properties, similar to the changes reported for rat zona glomerulosa cells in the presence of ACTH (23), our glomerulosa cells retained the capacity for a high level of aldosterone production.

While cultures with increasing enrichment of zona glomerulosa cells showed increasing levels of aldosterone, the increase was logarithmic with respect to, rather than directly proportional to, the number of these cells. One possible explanation for this logarithmic increase is that same-cell contact greatly amplifies the secretory capability of the cells. The influence of cell contact on steroid secretion has been demonstrated in cultures of human adrenocortical cells (predominantly zona fasciculata): 11-β-hydroxysteroids were seen at lower levels in cultures that contained mostly dispersed or single cells and at higher levels in cultures that contained primarily groups of
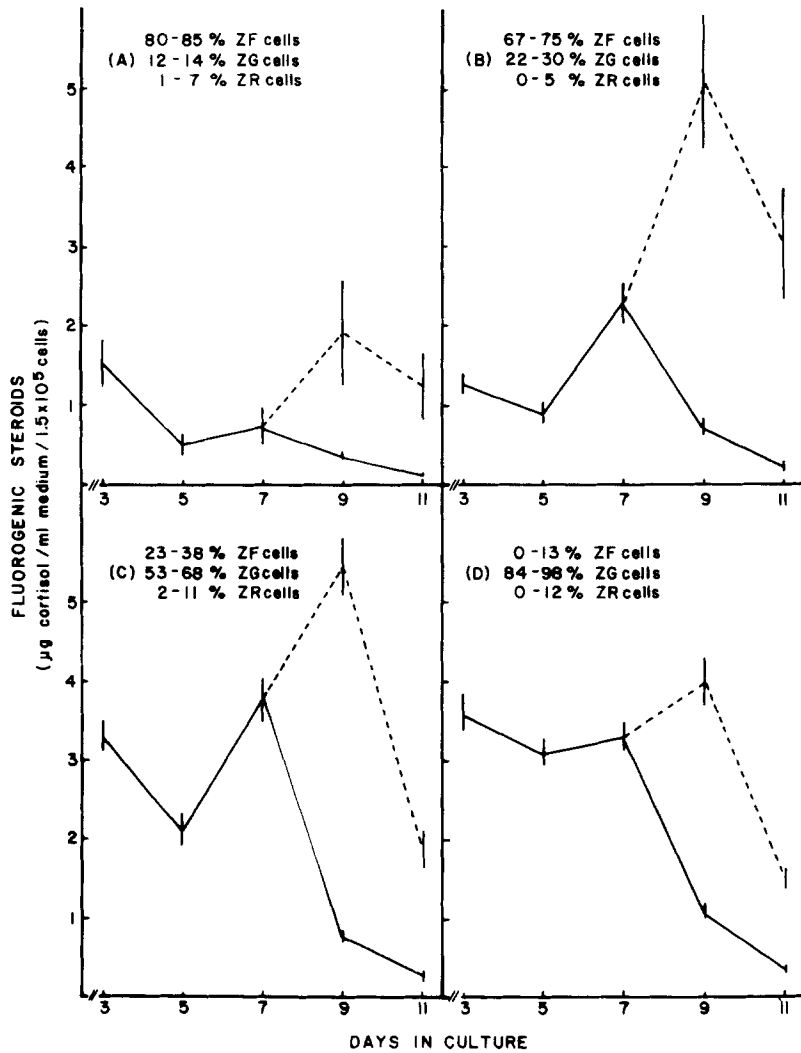
FIGURE 20 Levels of fluorogenic steroids present in cultures of adrenocortical cells where medium was changed every other day beginning on day 3. Solid line, control; dashed line, ACTH. ACTH (100 mU/ml) enhanced the levels in all cultures. In cultures enriched in zona fasciculata cells (A), the day 11 stimulated level remained higher than the day 7 basal level, but this was not the case for the zona glomerulosa enriched cultures (D). Levels are expressed as μg/ml of cortisol. Values are averages ± SE. Number of determinations: (A) days 3-7: 8-9, days 9-11: 5; (B) days 3 and 5: 41, days 7-11: 18-20; (C) days 3-7: 43-44, days 9 and 11: 22; (D) days 3-7: 119-125, days 9 and 11: 60-61.
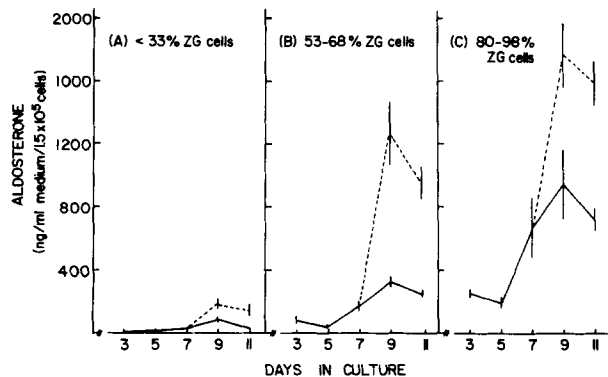

FIGURE 21 Aldosterone levels in cultures with different percentages of zona glomerulosa cells with (----) and without (——) ACTH treatment. Treatment was begun on day 7 for two consecutive 48-h periods. Note the increased level in all treated cultures. Cultures with 80-90% (C) zona glomerulosa cells had the highest absolute levels. Values represent averages ± SE. For each point of A and B, at least nine determinations were made; for each point of C at least 26 determinations were made.

cells (30). Contact between cells would be facilitated by increased cell density. In our study, cultures that have ~90% zona glomerulosa cells have a density of ~150 zona glomerulosa cells/mm² (35-mm dish), whereas cultures that have ~33%

zona glomerulosa cells have a density of ~50 zona glomerulosa cells/mm². In addition, microscopic observations showed that zona glomerulosa cells in the enriched cultures were in homogeneous groups, but in other cultures they were intermingled with zona fasciculata cells.

In response to ACTH, production of all steroids increased, but in differing ratios depending on the cell population. In zona fasciculata-rich cultures the ratio of ACTH vs. basal levels of fluorogenic steroids were consistently higher than those in zona glomerulosa-rich cultures. In addition, HPLC analysis showed that the ratio of cortisol to corticosterone increased with ACTH in zona fasciculata-enriched cultures more than it did in zona glomerulosa-rich cultures. Aldosterone production, in response to ACTH, achieved the highest absolute levels in those cultures which contained the highest number of zona glomerulosa cells. ACTH-stimulated increase of aldosterone production has also been demonstrated in rat zona glomerulosa cultures (23). ACTH-stimulated aldosterone levels in our cultures declined after day 9 when treatment was begun on day 7. Again similar results were obtained in ACTH-treated cultures of rat glomerulosa cells with treatment beginning on day 13 (22).

One persistent finding in this study was that mixed cultures of zona glomerulosa and zona fasciculata cells showed a higher output of both fluorogenic steroids and aldosterone, and a greater response of both to ACTH, than cultures enriched in either cell type. This may be due in part to interactions between

Table III

Fluorogenic Steroid Production in Response to Acute (2-h) ACTH Stimulation

Cell population		Fluorogenic Steroids, $\mu g/ml$ medium/0.75 × 10^5 cells							
		Day 0, 6 h	Day 0, 12 h	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
66% ZF, 32% ZG, 2% ZR	Basal	0.15 (0.92)[a] ±0.06 (±0.05)	0.14 (0.64)[b] ±0.01 (±0.06)	0.08 (0.99)[c] ±0.03 (±0.27)	0.02 ±0.02	0.10 ±0.02	0.07 ±0.03	0.22 ±0.06	0.07 ±0.04
	ACTH	0.67 (4.13)[a] ±0.01 (±0.31)	0.23 (3.56)[b] ±0.02 (±0.12)	0.32 (3.18)[c] ±0.03 (±0.22)	0.33 ±0.03	0.25 ±0.03	0.24 ±0.06	0.32 ±0.04	0.27 ±0.05
3% ZF, 96% ZG, 1% ZR	Basal	0.33 (2.60)[a] ±0.07 (±0.19)	0.42 (2.17)[b] ±0.04 (±0.08)	0.41 (2.34)[c] ±0.08 (±0.45)	0.06 ±0.02	0.21 ±0.04	0.17 ±0.03	0.22 ±0.03	0.13 ±0.02
	ACTH	0.43 (6.72)[a] ±0.09 (±1.00)	0.37 (7.04)[b] ±0.07 (±0.13)	0.58 (5.23)[c] ±0.07 (±0.50)	0.40 ±0.04	0.44 ±0.03	0.43 ±0.04	0.45 ±0.05	0.37 ±0.03

Cultures were treated daily for 2 h. On day 0, treatment was at either 6 or 12 h after plating. After each treatment, cultures were given media lacking ACTH. Values in parentheses denote levels of steroids detected in the subsequent 16[a], 10[b], or 22[c] h period in the absence of ACTH. Measurements represent averages of three to eight identically treated cultures.
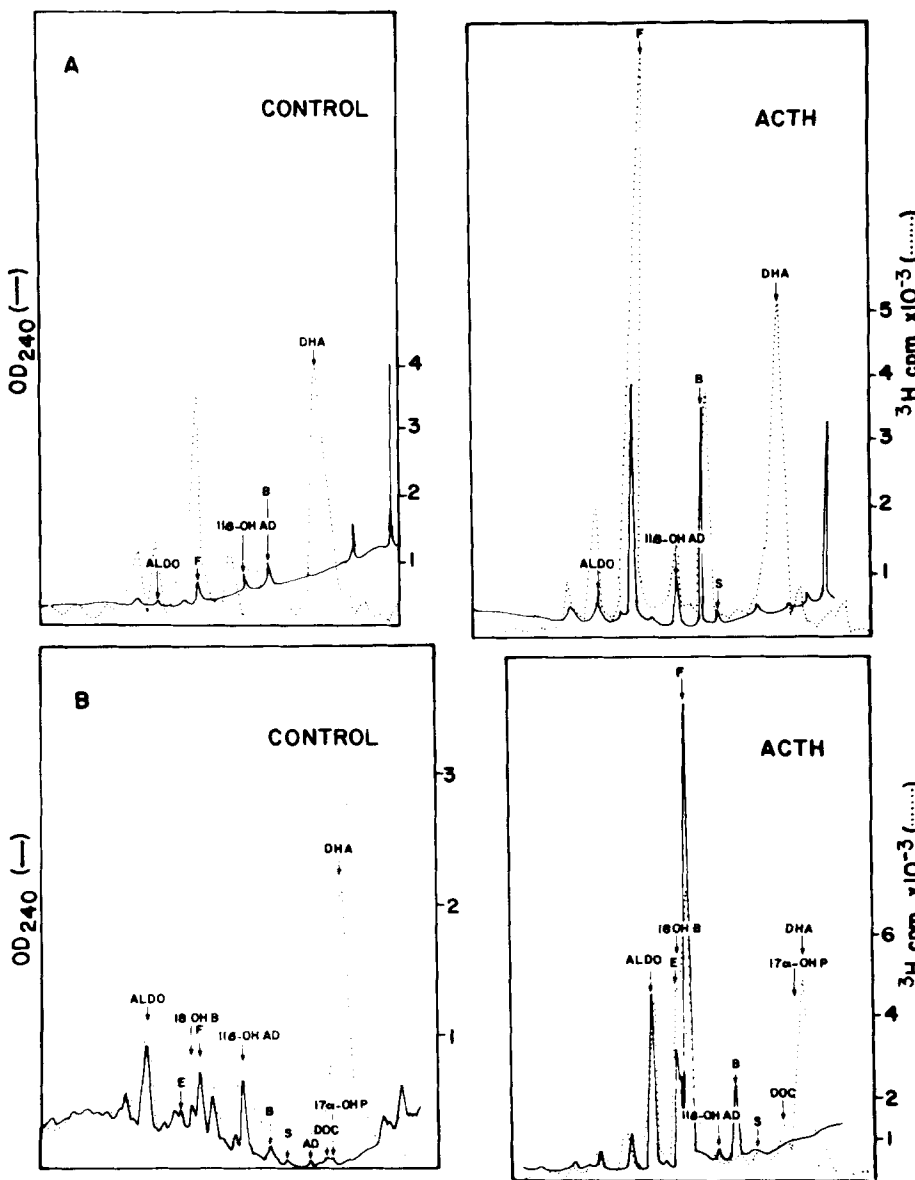

FIGURE 22 High pressure liquid chromatogram of steroids secreted by zona fasciculata-rich cultures (A) (80% zona fasciculata, 18% zona glomerulosa, 1% zona reticularis cells) and zona glomerulosa-rich cultures (B) (91% zona glomerulosa, 8% zona fasciculata, 1% zona reticularis cells) in absence and presence of ACTH. The cultures are incubated in fresh medium for 48 h, from day 7 to day 9 of culture, with or without ACTH (100 mU/ml) and in the presence of [7-^{3}H]pregnenolone (17.2 Ci/mmol; 1 μCi/plate). Medium was extracted with methylene chloride and the extracts dried and then redissolved in dioxane for separation by HPLC (——). Radioactivity was determined by scintillation counting of collected fractions (---). The retention times of steroid standards are indicated by the arrows: AD, androstenedione; ALDO, aldosterone; B, corticosterone; DHA, dehydroepiandrosterone; DOC, deoxycorticosterone; E, cortisone; F, cortisol; 11β-OH AD, 11-β-hydroxyandrostenedione; 18OH B, 18-hydroxycorticosterone; 17α-OH P, 17-α-hydroxypregnenolone; S, deoxycortisol. Attenuation: (A) control and +ACTH, 0.02 absorption units full scale (aufs); and (B) control, 0.01 aufs; +ACTH, 0.04 aufs.

zona fasciculata and zona glomerulosa cells which effected increased output of corticosterone/cortisol. Douglas et al. (15) stated that zona fasciculata cells present in cultures of zona glomerulosa cells may respond to ACTH with increased production of deoxycorticosterone and corticosterone and that these steroids can cause substrate-dependent stimulation of

aldosterone production by zona glomerulosa cells. Other studies have also demonstrated cooperative effects between the zona glomerulosa and zona fasciculata cells (37, 41). This finding underscores the need to obtain relatively pure populations of cells of study zonal specificity.

In our cultures ACTH-stimulated fluorogenic steroid production could be detected in zona glomerulosa–enriched cultures only after day 1, due to high basal secretion during the first day. This may indicate that these cells were stimulated by other factors in vivo and continued their response to stimulation in the first 24 h in vitro. This would be consistent with our observations of enhanced output for 22 h subsequent to treatment with ACTH for 2 h. Such observations emphasize the advantages of maintaining cells in vitro for periods of time before experimental treatment and of being able to retest the same population of cells over a period of time.

Angiotensin II could be stimulating zona glomerulosa cells in vivo; however, although aldosterone production could be detected in our cultured cells, they did not respond to angiotensin II. Several factors could account for this result. Angiotensin II receptors may have been altered during isolation and separation of the cells, the concentration of angiotensin II may not have been appropriate for the guinea pig or large enough to stimulate the isolated cells, and/or the cation (K^+ and Na^+) concentration may have been inappropriate. The importance of K^+ concentrations in the response to angiotensin II has been stressed in other studies using freshly isolated cells (3, 6, 10, 16, 17, 19, 26). In addition, sodium has been shown to affect the regulation of receptors and sensitivity to angiotensin II in freshly isolated cells (2). Further studies will be necessary to define angiotensin II responsiveness of zona glomerulosa cells in the guinea pig.

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