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A NEW COLUMN PERFUSION TECHNIQUE USING ISOLATED RAT ADRENAL CELLS TO STUDY ACTH AND DANAZOL

A Thesis

Presented to

The Faculty of the School of Sciences and Mathematics

Morehead State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Diane H. Johnson

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Accepted by the faculty of the School of Sciences and Mathematics, Morehead State University, in partial fulfillment of the requirements for the Master of Science degree.

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ABSTRACT

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It was found that this method could be successfully employed to study adrenal cell physiology, as all but 16.8% of the cells remained viable throughout continuous perfusion. The viable cells were shown to be stimulated effectively by 100 μU ACTH represented as an increase in basal corticosterone levels and inhibited by 100 μM of danazol represented as a decrease in basal

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INTRODUCTION

Various *in vitro* techniques have been employed to study adrenal physiology with emphasis toward increasing sensitivity to adrenocorticotropic hormone (ACTH). The *in vitro* method of Saffran and Schally (1955) based on the incubation of rat adrenal quarters, gives a dose-response relationship ranging from 10 to 400 mU/flask of ACTH. This relationship indicates a much lower sensitivity than was obtained by the *in vivo* method of Liddle (1962), in which a response to as little as 0.06 mU of ACTH injected into the whole animal was found. Possible explanations for the insensitivity of the adrenal slice preparation were that relatively few of its cells were in direct contact with ACTH, and that end-products remained in the incubate to suppress steroidogenesis. To overcome these difficulties and to provide the cellular integrity necessary for metabolic functions, continuous flow systems (superfusion) were devised (Saffran, *et al.*, 1967; Tait, *et al.*, 1967; Huibregtse and Ungar, 1970). The continous perfusion systems provided approximately a ten fold increase in sensitivity and provided "in line" adrenal quarters to more properly study the dynamics of adrenal stimulation and inhibition. However, the problem of cell contact to

the test substance was still evident.

The development of enzymatically isolated rat adrenal cell suspensions solved the problem of sensitivity by increasing cell surface area. Methods using collagenase (Kloppenborg, et al., 1968) or trypsin (Sayers, et al., 1971) were devised to provide a cell suspension in which all viable cells could be stimulated by the test substance. The enzymatically isolated cells were found to have up to a thousand fold increase in sensitivity to ACTH over the adrenal quarters (Nakamura, et al., 1971).

A comparison of incubated adrenal quarters, superfused adrenal quarters, and incubated isolated adrenal cells is shown in Figure 1, (Magrane, et al., 1974). It can be seen that the log dose 50 (LD_{50}, log dosage of ACTH stimulation which is half maximal) reflects the increase in sensitivity. The LD_{50} for adrenal quarter incubations was 50 mU/ml compared to 5 mU/ml for superfused adrenal quarters and 0.01 mU/ml for isolated adrenal cells. Thus the isolated adrenal cell preparation improved the sensitivity to ACTH by five thousand over the incubation of adrenal quarters. The problem of end-product influence was still evident.

Newer techniques to combine the sensitivity of

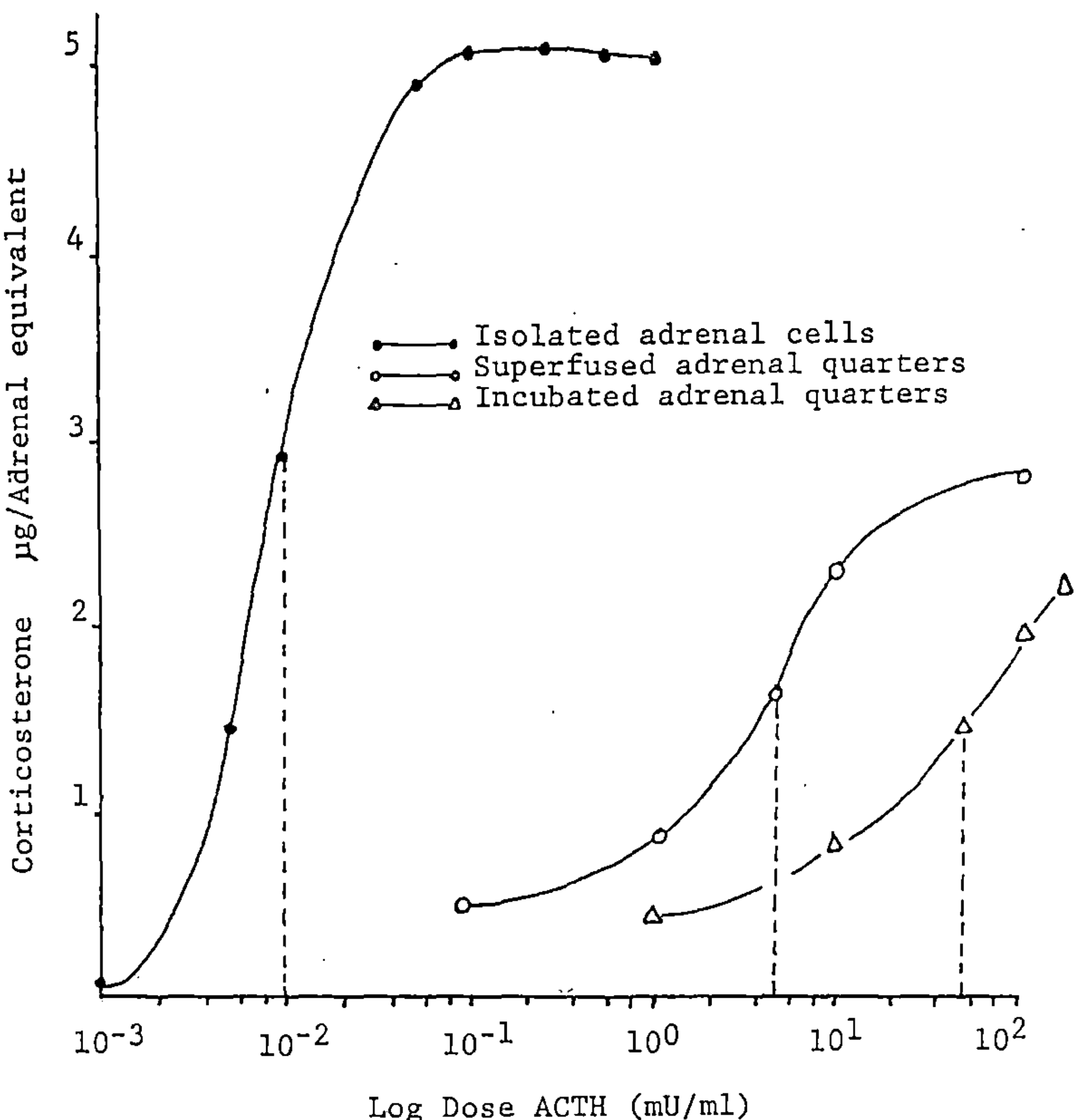

Fig. 1. Comparison of log dose stimulation by ACTH of various adrenal preparations (Magrane _et al_., 1974).

isolated cells with the advantages of superfusion were attempted. Using isolated rat adrenal cells, Lowry and McMarten (1974) devised a column perfusion testing method that was more controllable and provided more accurate physiological conditions. The cells were consistently responsive to concentrations of ACTH in the five to ten picogram/milliliter (pg/ml) range.

Column perfusion technique proved to have many advantages. It allowed for the removal of endogenous factors and end-products. The same cells could be tested by an inhibitor, rinsed, and then tested by a stimulant. Column perfusion was particularly advantageous for time-course studies, because consistent test substance concentrations and a constant flow rate could be maintained.

An improved method for superfusion of rat adrenal cells using a column apparatus was developed by McDougall and Williams (1978) which eliminated a kinetics problem found in the Lowry-McMarten system. However, for most laboratories these two column perfusion methods would be too time consuming and expensive to set up.

The need for an adrenal cell suspension combined with a continous flow system, that was economic and

simple to perform, would be a versatile tool in studies of adrenal metabolism. It would combine the advantages of cellular integrity with those of exposure of cell surfaces.

On the basis of chemical structure and biological activity, the hormones of the adrenal cortex may be grouped into four categories (Turner-Bagnara, 1976): 1) The 11-oxygenated corticosteroids that possess oxygen at carbon 11 and are especially potent in affecting carbohydrate and protein metabolism. The most important natural steroids of this class are cortisol, corticosterone, 11-dehydrocorticosterone and cortisone. 2) The corticoids that lack oxygen at position 11 have major effects on electrolyte and water metabolism. The most important mineral corticoids are 11-deoxycorticosterone (DOC) and 11-deoxycortisol. 3) Aldosterone, the most effective adrenocortical steroid in electrolyte metabolism, has an aldehyde group at C-18 instead of a methyl group. 4) Both androgens and estrogens, as well as progesterone are derived from the adrenal cortex. Since these sex hormones may arise from the adrenal, there is considerable overlapping between adrenocortical and gonadal functions.

All organs that synthesize steroid hormones, viz.,

adrenal cortices, testes, ovaries and placenta, possess the same enzyme systems. Although the enzymes that catalyze particular transformations at particular times may predominate in specific steroid hormone-producing tissues, the others are not completely absent. Acetate and cholesterol are known to be important precursors of the adrenal corticoids. The C-27 molecule cholesterol is the precursor of almost fifty different steroid compounds. The first step is the conversion of cholesterol to the C-21 molecule pregnenolone by a cytochrome P-450 enzyme of the mitochondria which requires NADPH and molecular oxygen and cleaves the side chain from cholesterol. The side chain cleavage step appears to be the rate limiting step in steroid biosynthesis according to Boyd, et al. (1978). (Figure 2.) Three enzyme systems, the 11-β, 17-α and 21-hydroxylases, catalyze the reactions leading to cortisol and corticosterone. Aldosterone may arise from cholesterol, progesterone, deoxycorticosterone or corticosterone, hydroxylation being accomplished at C-18. The conversion of androgens to estrogens involves hydroxylation at C-19. A separate hydroxylating system appears to be present for each position, and molecular oxygen and NADPH are used in the hydroxylations

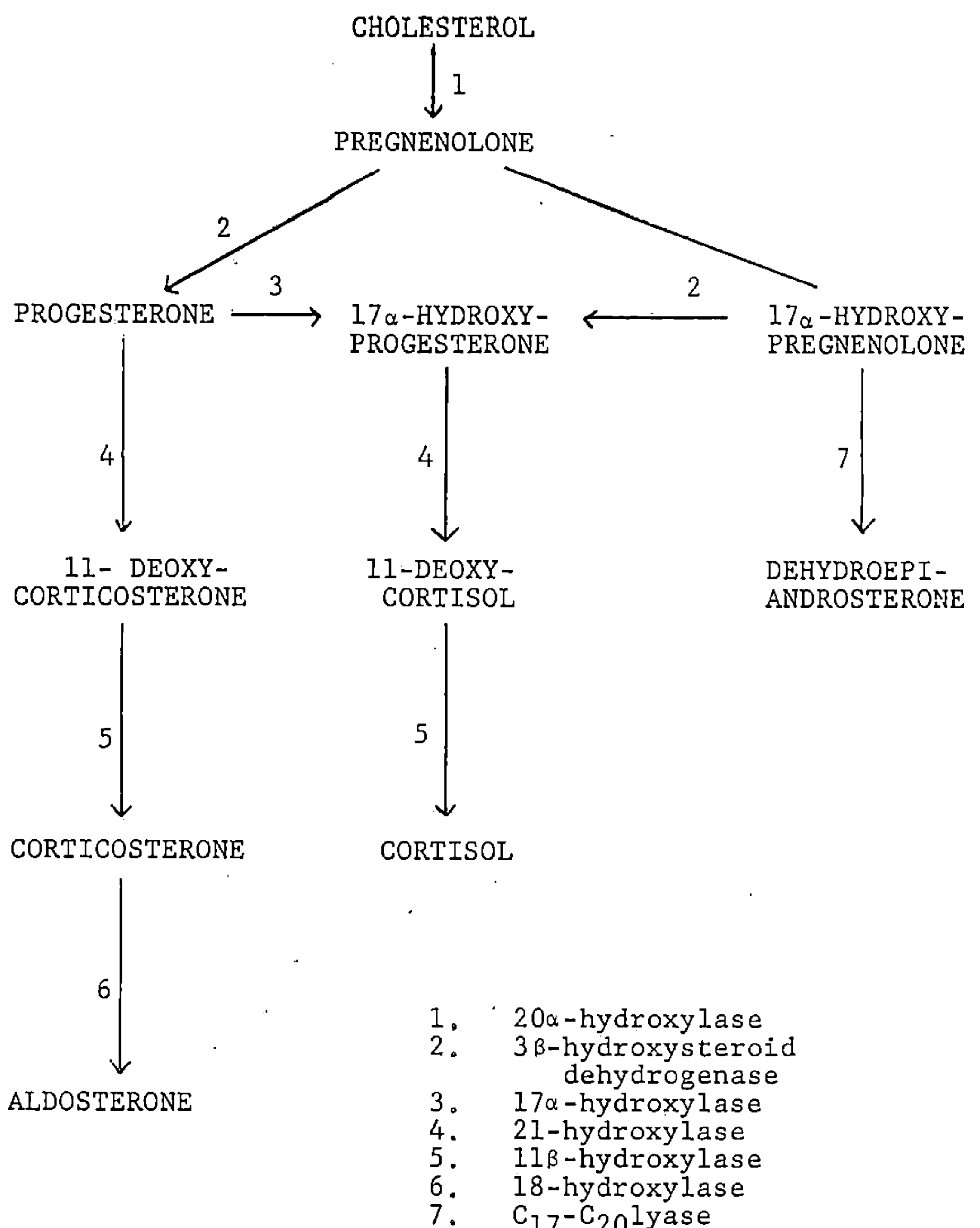

Fig. 2. Some Major Enzymes in the Biogenesis of Adrenal Cortical Hormones

(Greenberg, 1968). Since corticosterone levels were measured, only the pathways leading to the mineralcorticoids and the glucocorticoids will be discussed.

The conversion of pregnenolone to progesterone is catalyzed by 3β-hydroxysteroid dehydrogenase requiring NAD^+ as a hydro-acceptor and Δ^5-3-oxoisomerase which shifts the double bond from the 5 and 6 carbons to the 4 and 5 carbons. A 17α-hydroxylase associated with the cytoplasm converts progesterone to 17α-hydroxyprogesterone. This pathway is prevalent in humans but does not exist in rats. A microsomal 21-hydroxylase then converts 17α-progesterone to 11-deoxycortisol, a mineralcorticoid, which is then converted by the mitochondrial cytochrome P-450 11β-hydroxylase system to cortisol, the primary glucocorticoid in humans.

The primary pathway from progesterone in rats incorporates a 21-hydroxylase to produce deoxycorticosterone (DOC), a mineralcorticoid comparable to 11-deoxycortisol. DOC is then converted by the 11β-hydroxylase to corticosterone, the primary glucocorticoid in rats (Boyd, <u>et</u> <u>al</u>., 1978).

The 3β-hydroxysteroid dehydrogenase, 11β-hydroxylase, and 21-hydroxylase activity occur throughout the

adrenal cortex. Sabatini and Roberts (1961) found that the 18-oxygenation to aldosterone is located only in the outermost zona glomerulosa, and the 17-hydroxylase activity is localized in the inner zona fasciculata or the zona reticularis. It has been shown that the zona glomerulosa and zona reticularis are independent of each other (Turner and Bagnara, 1976). According to a study by Watanuki, et al., (1978), there is evidence that the 11β-hydroxylase and 18-hydroxylase activities, which are part of the cytochrome P-450 complex, are actually catalyzed by only one enzyme (Figure 3).

The regulation of synthesis and secretion of these various steroid groups is specified by feedback mechanisms that affect the cytophysiology of the adrenal cortex. ACTH is the main hormone that stimulates the adrenocortical secretion. Like most of the peptide hormones, the action of ACTH appears to be mediated by the "second messenger," cyclic AMP. It is thought that the activity of adenylate cyclase at the cell membrane of adrenocortical cells is stimulated by ACTH, leading to an increased conversion of ATP to cyclic AMP. This increased concentration of cAMP within the cell is thought to accelerate the conversion of cholesterol to pregnenolone, a basic and rate-limiting step in adrenal

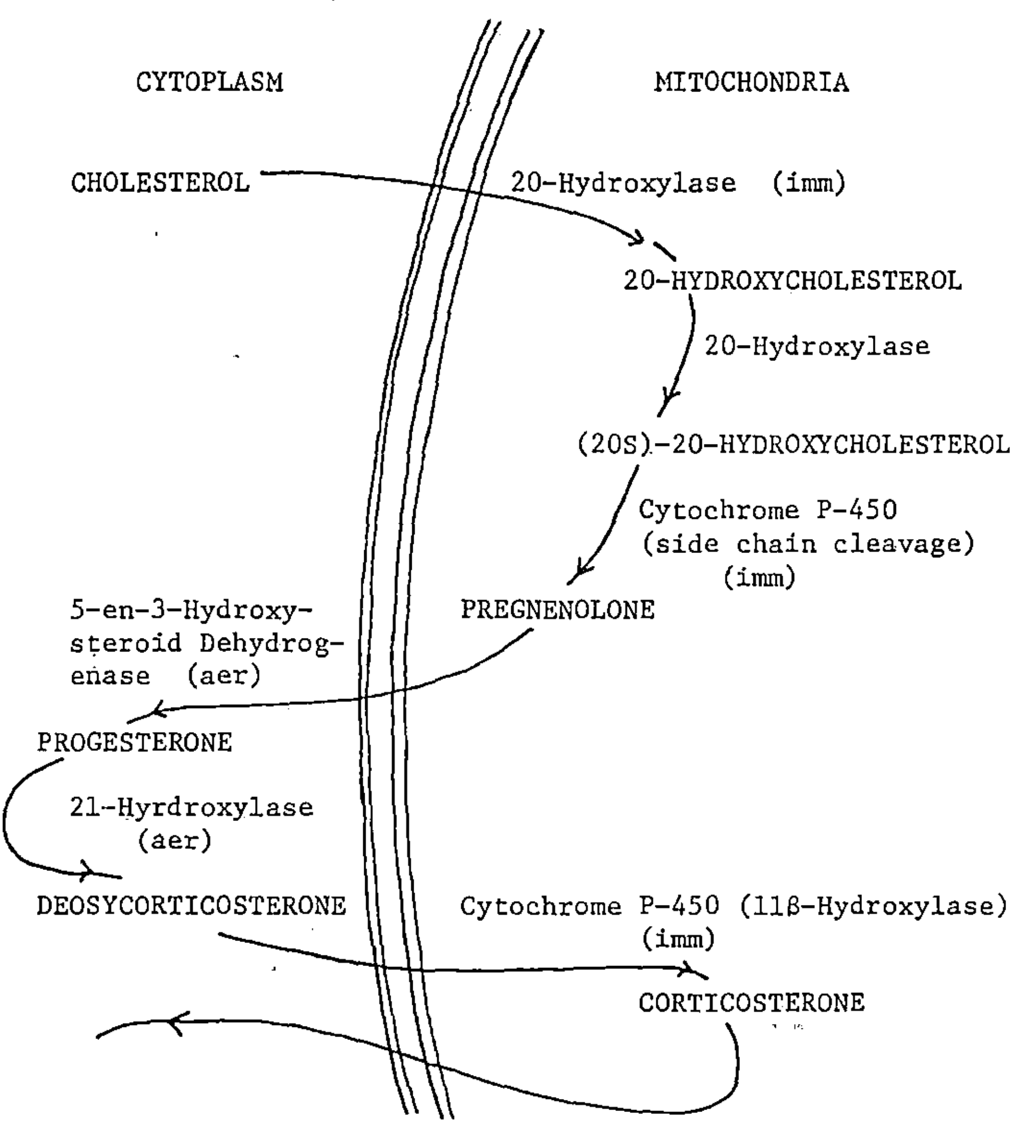

Figure 3. Biosynthesis of corticosterone from cholesterol (Menke, 1978)

imm = inner mitochondrial membrane
aer = agranular endoplasmic reticulum

steroidogenesis (Turner and Bagnara, 1976, and Sutherland, *et al.*, 1968). The question of how cAMP initiates this basic step in steroidogenesis is as yet unresolved. In an *in vitro* system, it has been shown that the rapid effects of ACTH and cAMP on steroid biosynthesis are accompanied by rapid protein synthesis, thus cAMP's action may be through selective effects on protein synthesis (Grower and Bransome, 1970).

Danazol, a heterocyclic steroid related chemically to 17-ethinyltestosterone (ethisterone) was synthesized at Sterling-Winthrop Research Institute by Manson, *et al.*, in 1963. Danocrine, brand name of danazol, is a synthetic androgen with the chemical name, 17α-pregna-2,4-dien-20[yno]- 2,3-d -isoxazol-17-ol, and the structural formula shown in Figure 4. The widest

OH
C≡H
N
O

Figure 4. Structure of danazol.

clinical application of danazol, as cited by Dmowski (1979), has been for the treatment of endometriosis. Currently, danazol is approved by the Food and Drug Administration only for use in endometriosis at the highest dosage of eight-hundred milligram/day (800 mg/day).

The pharmacology of danazol is not completely understood. In a review by Barbieri and Ryan (1981), the effects of danazol are summarized as follows: 1) danazol prevents the midcycle surge of lutenizing hormone (LH) and follicle stimulating hormone (FSH); 2) danazol does not significantly suppress basal LH or FSH in gonadally intact human beings; 3) in castrated animals danazol can prevent the compensatory increase in LH and FSH; 4) danazol binds to androgen, progesterone, and glucocorticoid receptors; 5) the danazol-androgen receptor complex can translocate into the nucleus and initiate androgen-specific RNA synthesis; 6) the danazol-progesterone receptor complex translocated poorly and can initiate minimal, if any, RNA synthesis; 7) danazol does not bind to intracellular estrogen receptors; 8) danazol binds to sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG); 9) danazol inhibits cholesterol cleavage enzyme,

3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 17,20-lyase, 17α-hydroxylase, 11β-hydroxylase, and 21-hydroxylase; 10) danazol does not inhibit aromatase; 11) danazol increases the metabolic clearance rate (MCR) of progesterone; and 12) metabolites of danazol are hormonally active.

The complex pharmacology of danazol makes it difficult to accept the statement that danazol is a "selective antigonadotropin with minimal androgenic effects and no other hormomal properties," as stated in the Physician's Desk Reference (1979). It may be more suitable to refer to danazol as a synthetic steroid with androgenic and mixed progestational-antiprogestational effects which produces anovulation and low levels of circulating estrogen and progesterone by actions at the hypothalamic, pituitary, and ovarian levels, as suggested by Barbieri and Ryan, 1981.

Reviewing _in vitro_ experiments, danazol has been shown to inhibit multiple enzymes of steroidogenesis, including cholesterol cleavage enzyme, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 17,20-lyase, 11β-hydroxylase, and 21-hydroxylase. Biochemical analysis suggests that danazol competitively inhibits the previously mentioned

enzymes by binding to the active site for the steroid substrate. It is likely that danazol modifies ovarian steroidogenesis either by inhibiting these enzymes or by binding to ovarian androgen receptors and blocking the synthesis of proteins essential to steroidogenesis (Barbieri, et al., 1977).

Studies by Stillman, et al., 1980, suggest that danazol may partially inhibit human adrenal 3β-hydroxysteroid dehydrogenase and 11β-hydroxylase in vivo. Danazol administration, in this study, resulted in no significant change in baseline cortisol concentration, but resulted in a significant increase in progesterone, dehydroepiandrosterone, and androstenedione concentrations. The administration of ACTH during the danazol treatment period resulted in greater increments in pregnenolone and 11-deoxycortisol and smaller increments in progesterone and cortisol than those seen in the control peroid. These findings suggest that danazol produces a partial inhibition of 3 β-hydroxysteroid dehydrogenase and 11β-hydroxylase in vivo. As the suggested mechanism for blocking ovarian steroidogenesis is by means of binding to an ovarian androgen receptor, this mechanism may hold true in the adrenal gland with the discovery of an adrenal androgen

receptor (Rifka, *et al.*, 1978).

It is the purpose of this research to provide a simple technique which can readily yield accurate data on responses of isolated rat adrenal cells to ACTH, a stimulator and danazol, a suggested inhibitor of steroidogenesis. Column perfusion technique can be used to mimic physiologic conditions while allowing a time-course study to be performed, so that accurate comparisons concerning steroidogenesis can be made between unstimulated, ACTH stimulated, and danazol inhibited cells by measuring the corticosterone levels of the effluent. Based on viability studies of the cells and study of the experimental design and procedure, it can be shown that this rat adrenal cell suspension and column set-up using a peristaltic pump can be versatile and valuable tool in future studies of this nature. It is worthwhile to note that the cost and simplicity of this system would not be prohibitive to most laboratories.

MATERIALS AND METHODS

Animal Care

Adult female Sprague-Dawley rats, weighing 200-300 grams were housed in the animal quarters at 23°C under a lighting regimen of fourteen hours light and ten hours dark. The rats were fed Purina Laboratory Rat Chow (Ralston-Purina Co.) and had access to food and water *ad libitum*.

Adrenal Cell Isolation

All glassware used in the isolation procedure was siliconized with Siliclad (Clay-Adams), rinsed in distilled water and dried in an oven at 60°C.

Rats were stunned, decapitated and exsanguated within ten seconds after their removal from the animal quarters to minimize ACTH release into the bloodstream. The adrenals were removed within one minute and placed on a saline-moistened filter paper in a petri dish on ice. After all adrenals had been collected, they were trimmed of excess fat with a razor blade and weighed to the nearest milligram (mg).

Isolated adrenal cells were prepared by a modification of the collagenase method of Kloppenborg, *et al.*, 1968. The adrenals were placed on a glass plate and eighthed using a razor blade. Each pair was added

to a 25 ml Erlenmeyer flask containing the enzyme digestant solution at the following ratios per pair of adrenals: 1 ml Krebs Ringer Bicarbonate buffer (Umbreit, Burriss and Stauffer, 1964) to which 200 mg % glucose was added (KRBG), 40 mg of bovine serum albumin (BSA, Sigma Biochemicals, Inc.), 5 mg collagenase (Worthington Biochemical Corp., Type I, 133 Unit/mg), and 0.05 mg Deoxyribonuclease I (DNAase, Sigma Biochemicals, Inc.). The flasks were incubated forty-five minutes at 37 °C under an atmosphere of 95:5, $O_2:CO_2$ in a Dubnoff Metabolic Shaking Incubator (Precision Instruments). Using a nine inch Pasteur pipet, the cells were dispersed with fifty repeated excursions. The cell isolate was filtered through a stainless steel mesh screen, pore size one-hundred microns, into a cold 15 ml polystyrene conical centrifuge tube. The incubation flask was rinsed with 1.0 ml of KRBG to remove adhering cells and filtered as above. The filtered digestant was centrifuged for ten minutes at 100 x g on a table-top centrifuge. The supernatant was aspirated and discarded with a Pasteur pipet and the cell pellet was washed with 1 ml of KRBG and resuspended. The digestant was recentrifuged at 100 x g for ten minutes. The supernatant was aspirated

and discarded as above, and the cell pellet was then suspended in the media used for the column perfusion (see Column Perfusion Technique) and placed on ice until needed.

Cell counts were taken before and after perfusion to determine cell viability and the number of cells per adrenal equivalent. One drop of methylene blue was added to a 0.5 ml aliquot of isolated cells, and the cells were counted using a hemocytometer.

Construction of Apparatus

The column apparatus was prepared by first boring a 1.2 millimeter (mm) hole through the rubber tip on the end of the plunger of a 2½ cc plastic, disposable syringe. Tygon microbore tubing (1.27 mm by 2.22 mm) was inserted from the bottom, up and through the tip until one end of the tube was flush with the rubber tip (see Figure 5). Secondly, an 11 mm stainless steel disc-shaped screen, 100 μ mesh, was cut using a size five cork borer. The screen was placed over the tip of the plunger to prevent cell loss during perfusion. The plunger and screen were inserted into the syringe until the screen and top of the plunger were at the 2 cc mark. Another column was prepared in the same manner so that two perfusions could be performed simultaneously.

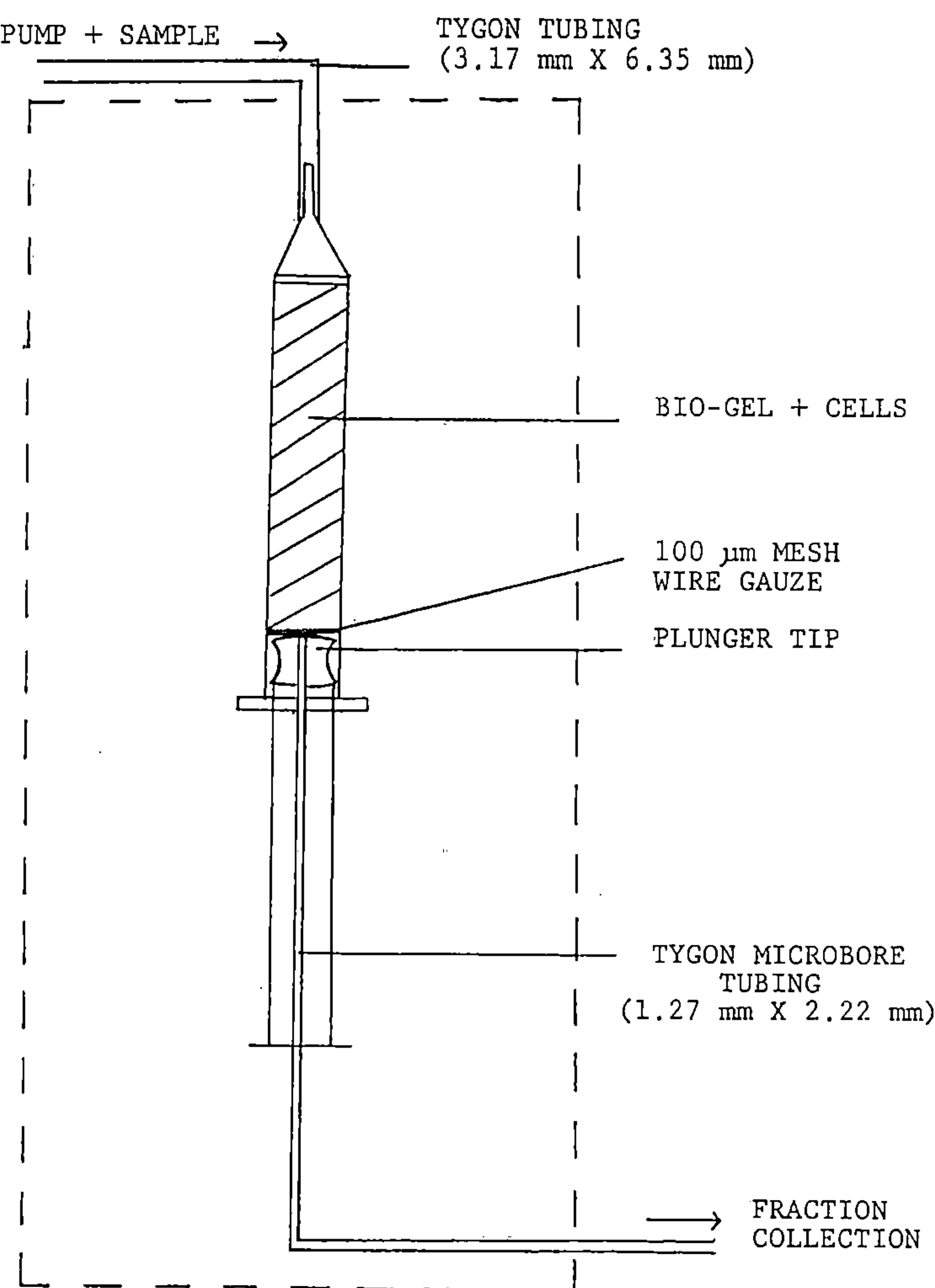

Fig. 5. Column Perfusion Apparatus

Column gels were prepared by adding 0.5 g of Bio-Gel P-2 (Bio Rad Laboratories, 50-100 mesh) to 2.5 ml of 0.9% sodium chloride and allowed to swell for fifteen minutes in a 5 ml disposable beaker. Using a seven inch Pasteur pipet, 0.5 ml of the swollen Bio-Gel beads were placed in the bottom of the column apparatus. The adrenal cell suspension (see Column Perfusion Technique) was mixed with the remaining Bio-Gel and placed in the column apparatus using a seven inch Pasteur pipet. The beaker of Bio-Gel and cells was rinsed twice .

Each column was then connected to a clamp attached to a ringstand. A larger gauge Tygon tubing (3.17 mm by 6.35 mm) was inserted on the tip of the clamped, upright column and the other end was connected to the peristaltic pump (Model 203, Scientific Industries). The columns on the ringstand were placed into a Labline incubator with enough water at 37 °C to completely cover the columns. The microbore tubing extending from inside the column was placed into a labelled 6 ml disposable glass tube.

<u>Column Perfusion Technique</u>

Collagenase isolated rat adrenal cells were resuspended after the second spin in the following

buffer for perfusion as modified from Lowry (1974): KRBG (1 ml), BSA (5 mg), 0.1% penicillin (25 m units/ml) and streptomycin (25 g/ml, Sigma Chemical Co.). The media was allowed to equilibrate for one hour prior to the addition of the cells.

The peristaltic pump was primed with KRBG to remove air bubbles from the hose prior to connection to the column apparatus. The two columns were connected to the primed pump and the Bio-Gel-cell suspension was packed down under a flow of 1 ml/min of perfusion media for ten minutes at 37°C in a Labline incubator (see Figure 6). This allowed for the removal of endogenous adrenal products, so that a more accurate effect on steroidogenesis could be measured.

Experimental Design

Direct ACTH stimulation at the concentration of 100 μU/ml of perfusion media was studied by collecting samples every 5 minutes for 35 minutes in six ml disposable tubes. ACTH (ACTHar, 40 U/vial injectible, Armour Pharmaceuticals, Inc.) was added at basal time five minutes. Three separate experimental runs were performed against an unstimulated control for comparison so that a mean stimulation could be ascertained. Sample tubes were lableled, covered with parafilm and frozen at

PERISTALTIC PUMP

in

out

out

in

CONTROL
MEDIA

TESTING
MEDIA

WATER
LINE

COLUMNS

C

COLLECTION
TUBES

TIMER

LABLINE INCUBATOR (37°C)

Fig. 6. Completed Column Perfusion Apparatus

-20°C.

Next, direct danazol effect was studied at total concentrations of danazol (Sterling-Winthrop Research Institute) in perfusion media of 10 μM, and 100 μM. Danazol in absolute ethanol was added at the basal time five minutes and three separate experimental runs were performed against an untreated control for each concentration so that a mean effectiveness could be determined. Samples were collected in 6 ml disposable tubes for thirty-five minutes at 5 ml sample/5 min. Sample tubes were labeled, covered with parafilm and frozen at -20° C.

Finally, the effectiveness of danazol against direct ACTH stimulation was studied. Danazol at total concentrations in perfusion media of 10 μM and 100 μM were added at basal time 0 and ACTH at 100 μU/ml added at five minutes. Sample tubes were treated as above.

<u>Fluorimetric Analysis for Corticosterone</u>

A corticosterone standard of 250 mg/ml was prepared in absolute ethanol for comparison with test samples. Aliquots of 2.0, 1.5, 1.0, and 0.5 ml of the corticosterone standard were added to 15 ml conical centrifuge tubes, and each was brought up to 2.0 ml with distilled water. A tube containing 2.0 ml of distilled

water served as a blank. Parallel aliquots of 2.0 ml of the samples were also prepared. A 5.0 ml addition of methylene dichloride ($MeCl_2$) was pipeted into each standard and sample tube to extract the corticosterone. The tubes were vortexed for fifteen seconds and 2.0 ml of the $MeCl_2$ extract were transferred to 15 ml round bottom tubes. A fluorescent reagent was prepared fresh each day and consisted of 65% concentrated sulfuric acid and 35% absolute ethanol. To each tube, 2.0 ml of fluorescent reagent was added, and the tubes were vortexed for fifteen seconds. The tubes containing the $MeCl_2$ and fluorescent reagent were allowed to develop for sixty minutes. Readings were taken as percent transmission on an Aminco-Bowman Spectrophotofluorometer (courtesy of the University of Kentucky) at the excitation level of 468 nm and emission of 520 nm. The sample tube were read against a standard curve prepared with the blank set at 0% transmission and the 2.0 ml standard set at 100%.

Statistical Analysis

Data was analyzed by the student's t test.

RESULTS

It can be seen from Table 1 that the total counts averaged for all assays before perfusion was 364,000 viable cells/cubic millimeter (mm^3). The mean total cell count taken after perfusion showed 303,000 viable cells/mm^3. Thus, the change in cell counts was 61,000 cells/mm^3, indicating a 16.8% reduction in the number of viable cells during continous perfusion.

Evidence for cell viablity in the columns was demonstrated by the ability for adrenal cells to respond to 100 μU ACTH (Figure 7). There was no significant difference between the control and experimental columns in their basal release of corticosterone at the 5 minute level. However, a significant difference was obtained ($P<.02$) five minutes after the addition of ACTH. ACTH stimulated corticosterone release at highly significant levels throughout the length of perfusion.

Danazol showed a reduction in basal release of corticosterone in isolated rat adrenal cells in column perfusion (Figure 8). The 10 μM concentration of danazol did not significantly lower basal corticosterone levels. The 100 μM concentration of danazol reduced corticosterone release which was significant thirty minutes after the addition of danazol ($P<.01$).

TABLE 1.

CELL VIABILITY

TREATMENT N=3	CELL COUNT BEFORE PERFUSION (X $10^5/mm^3$)	CELL COUNT AFTER PERFUSION (X 10^5/mm)	CHANGE IN COUNTS (X $10^5/mm^3$)
CONTROLS	4.13	3.44	0.69
100 µU ACTH	2.80	2.33	0.47
1 µM DANAZOL	5.20	4.33	0.87
10 µM DANAZOL	3.88	3.23	0.65
100 µM DANAZOL	3.32	2.76	0.56
100 µU ACTH + 10 µM DANAZOL	3.32	2.76	0.56
100 µU ACTH + 100 µM DANAZOL	2.80	2.33	0.47
TOTALS (mean ± S.D.)	3.64 ± 2.49	3.03 ± 0.50	0.61 ± 0.02

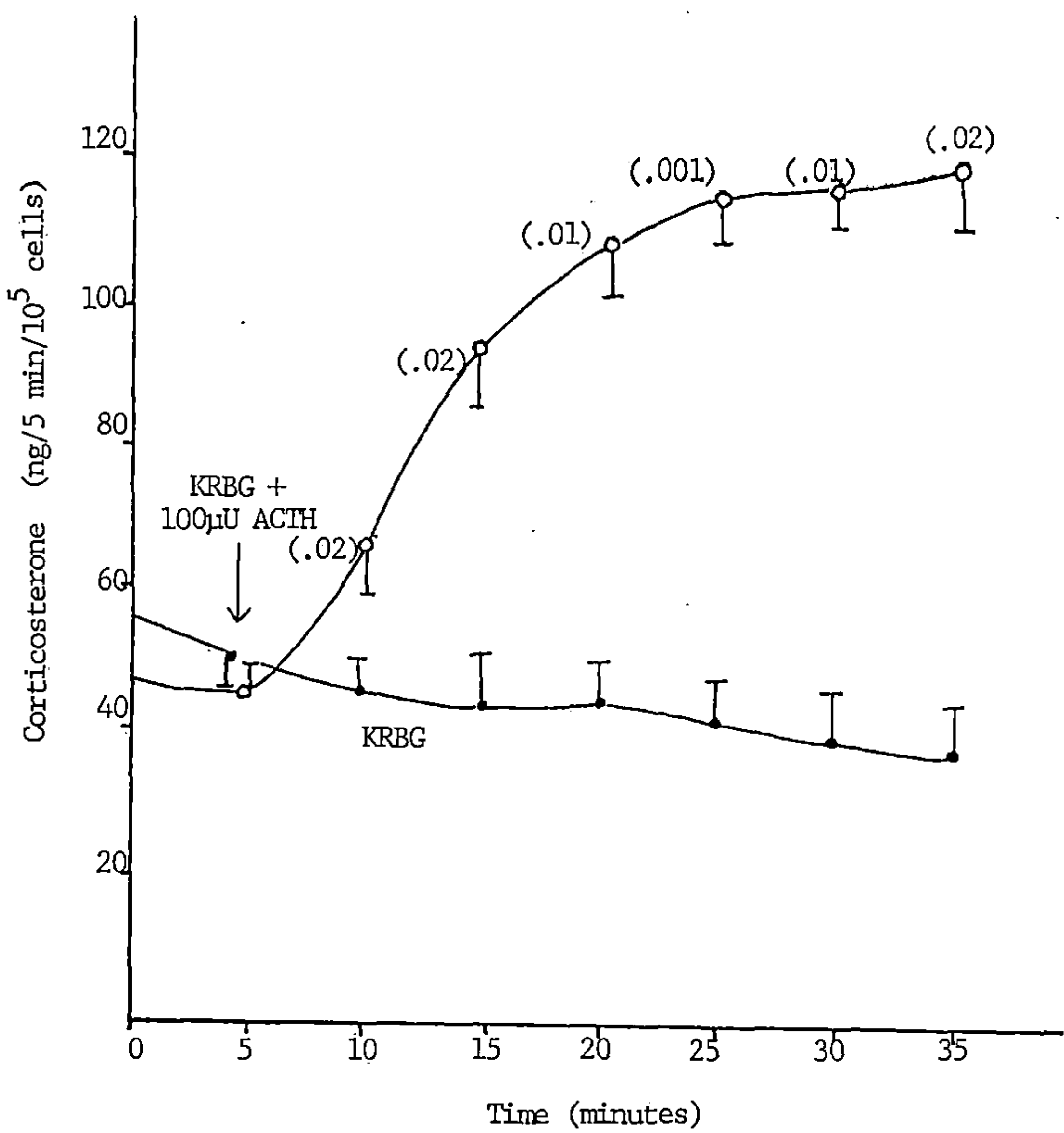

Figure 7. Stimulation by ACTH of column perfused adrenal cells. Each point represents a mean of three assays $\pm$ standard deviation. Numbers in parenthesis indicate probability levels compared to controls.

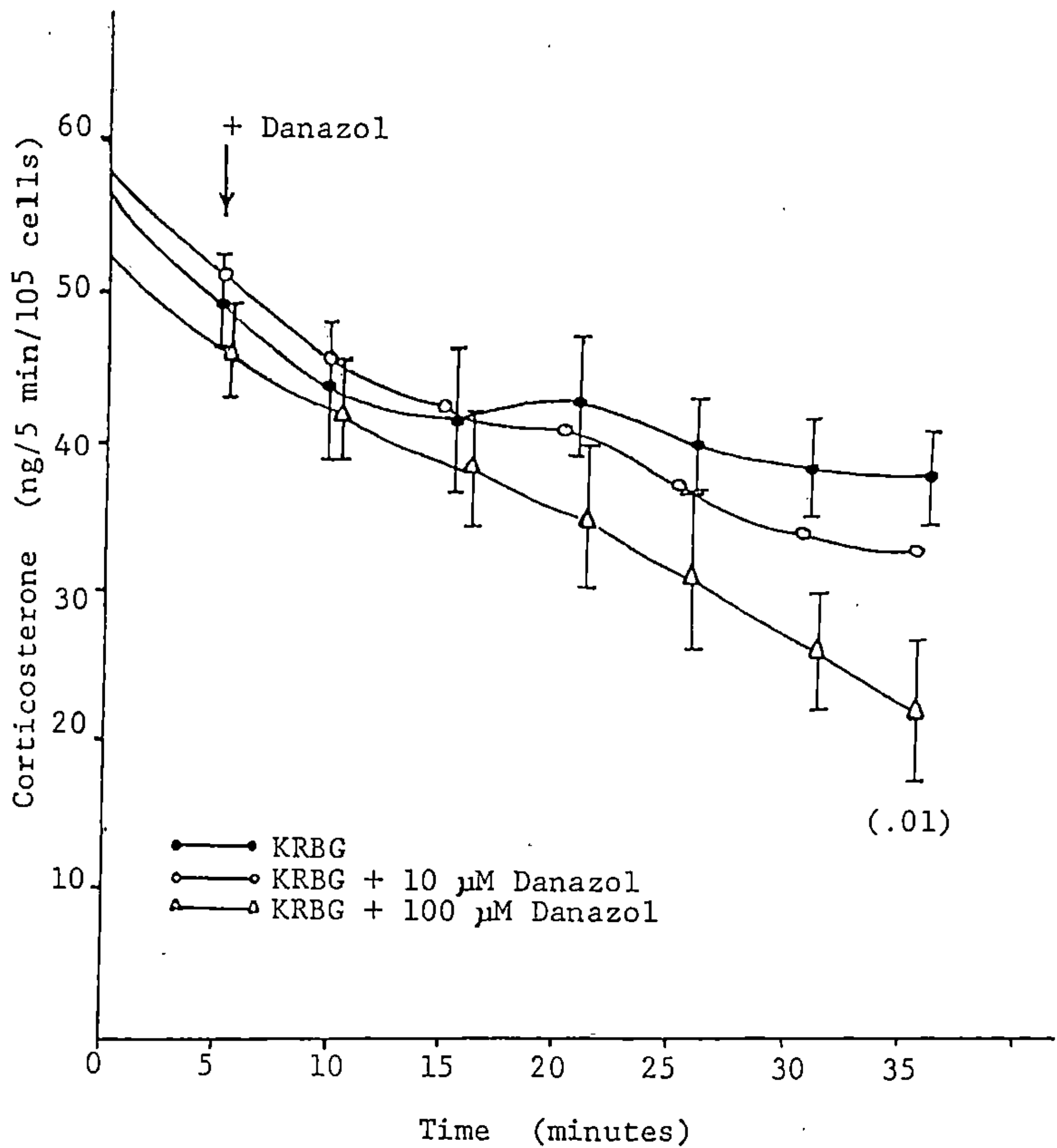

Figure 8. Danazol inhibition of basal corticosterone release. Each point represents a mean of 3 assays ± standard deviation. Number in parenthesis indicates probability levels compared to controls.

The effect of danazol inhibition on ACTH stimulated steroidogenesis is shown in Figure 9. The 10 μM dose of danazol did not inhibit the stimulatory effect of ACTH (100 μU), a concentration that significantly elevated corticosterone release ten minutes after addition. Increasing the dose of danazol to 100 μM resulted in a more pronounced but not complete inhibition of 100 μU ACTH. The greatest difference was seen 20 minutes after addition of ACTH.

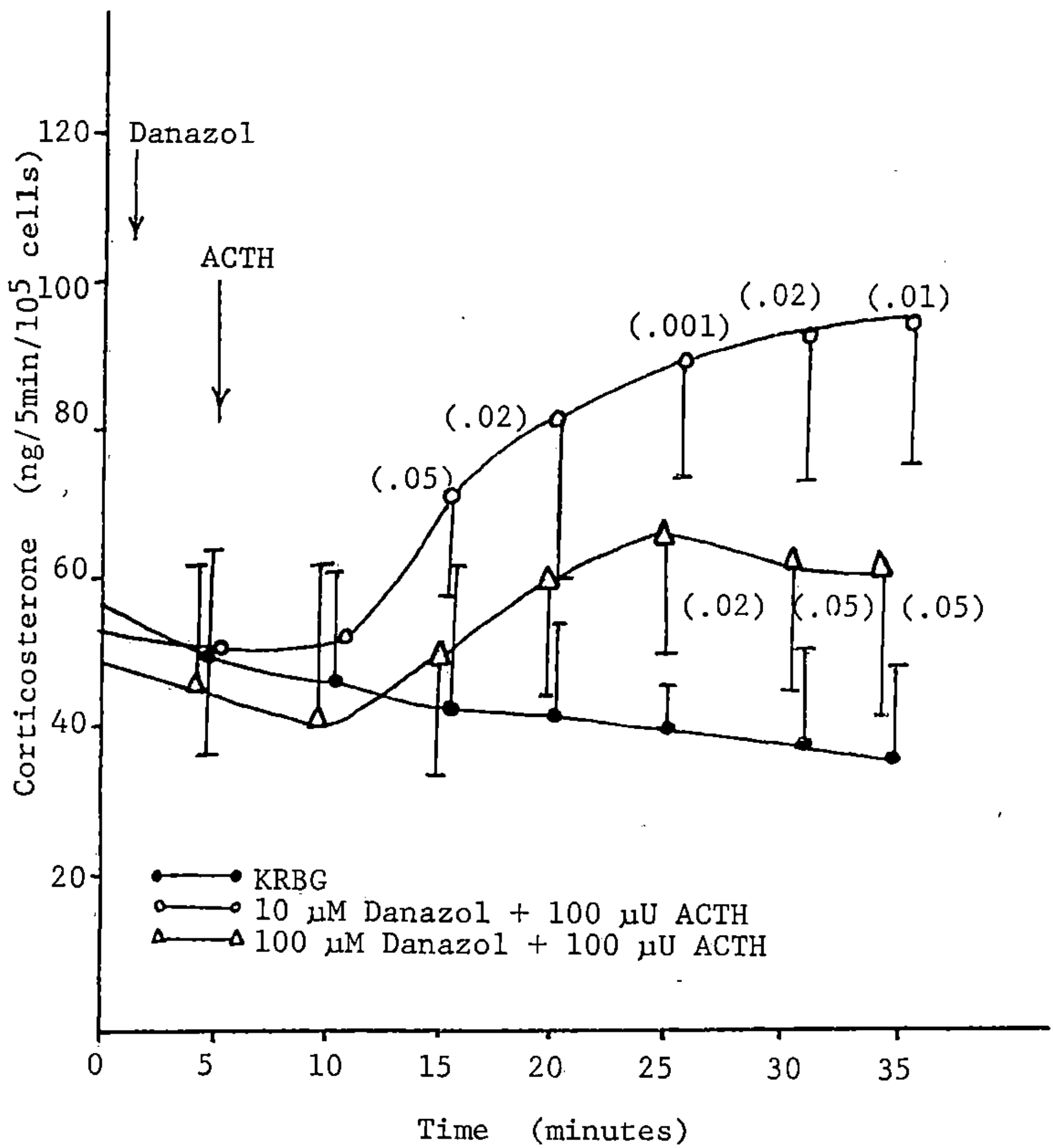

Figure 9. Danazol inhibition of ACTH stimulation of column perfused adrenal cells. Each point is a mean of three assays ± standard deviation. Number in parenthesis indicates probability level compared to controls.

DISCUSSION

Numerous techniques have been developed for analysis of adrenal physiology *in vitro*. Adrenal quarters were incubated (Saffran and Schally, 1955), but nonphysiological amounts of ACTH proved necessary for stimulation. In addition, this incubation system allowed for end-product build up which could alter normal metabolism. Continuous perfusion systems using adrenal quarters were devised (Saffran, *et al.*, 1967; Tait, *et al.*, 1967; Huibregtse and Ungar, 1970) which succeeded in removing the end-product, but it only increased sensitivity ten fold due to the inability of ACTH in contacting the cells. The problem of sensitivity to ACTH was overcome after development of an isolated cell system (Kloppenborg, *et al.*, 1968; Sayers, *et al.*, 1971). However, isolated cell incubations again faced the problem of feedback of the end-products. To alleviate these drawbacks Lowry-McMartin (1974) and McDougall and Williams (1978) developed column perfusion systems using isolated rat adrenal cells, which proved to be too expensive and time-consuming for most laboratories.

A simple and inexpensive system for testing compounds on isolated rat adrenal cells would be very

useful in studies concerning adrenal metabolism. The column perfusion system developed in this research proved to be functional with minimal loss of cells (16.8%). Some of this loss may have been due to difficulty in counting the cells in the gel media, therefore the reduction in the cell counts may not reflect true cell loss.

ACTH has been proven to be effective in stimulation of steroidogenesis in isolated rat adrenal cells. A time lag of two minutes before increase in steroid production is characteristic of ACTH's mode of action (Sutherland, 1968). The isolated rat adrenal cells in this research showed a time lag of five minutes after the introduction of ACTH into the system before a significant increase in corticosterone was observed (see Figure 7). This may be accounted by the one minute time lag for ACTH to reach the cells, and the characteristic two minute lag after ACTH initially stimulated the cells. The fact that ACTH stimulated cells responded over the full 30 minutes indicated that they are viable and do not become exhausted.

Danazol has been shown to inhibit steroidogenesis *in vitro* (Barbieri, *et al.*, 1977) by several possible means which was somewhat contradictory to evidence cited

by Wentz, *et al.*, 1975. However, *in vivo* studies using danazol (Stillman, *et al.*, 1980) showed inhibition of multiple enzymes of steroidogenesis. Therefore, due to the inconclusiveness of the evidence of danazol's pharmacology and its effect on the adrenal gland more research seems necessary. This research showed that there was no early fall in basal corticosteroid levels due to the lower dose of danazol (10 μ M), when compared to the basal release in unstimulated cells (see Figure 8). However, a significant drop in corticosterone levels was noted thirty minutes after the addition of 100 μU danazol, suggesting that higher doses of danazol are capable of inhibiting steroidogenesis *in vitro*. Because adrenal cells were releasing steroids at basal levels, it would not have been expected that an inhibitor would have significantly reduced steroid release to any great extent. A clearer understanding of danazol's inhibition of steroid synthesis could be gained by simultaneous treatment with ACTH stimulated adrenal cells.

The fact that a 10 μM dose of danazol, added 5 minutes prior to ACTH stimulation, did not inhibit steroidogenesis might indicate that the dosage was too small. A dose ten times greater was effective in

reducing steroidogenesis until 20 minutes after the addition of ACTH. This retardation of corticosterone release would suggest danazol to be mildly inhibitory. This supported the evidence of Barbieri, et al., 1977, that danazol can inhibit the multiple enzyme systems of steroidogenesis by binding to androgen receptors. These receptors were shown to be present in rat adrenal cells by Rifka, et al., 1978. Data reported by Stillman (1980) in humans suggest danazol administration did not alter basal cortisol levels, but did significantly increase progesterone, dehydroepiandrosterone and androstendione. This suggested a block of 3β-hydroxysteroid dehydrogenase and 11β-hydroxylase (see Figure 2). The results shown on this research of isolated perfused adrenal cells concurred with the partial inhibition of these enzymes.

I have shown that a simple column perfusion system using isolated rat adrenal cells can be developed that is also economical. Viability of cells was shown by uptake of methylene blue. The cells proved to be physiologically viable by responding to ACTH. Evidence suggested that danazol is a suspected direct inhibitor of steroidogenesis, and partially inhibits the stimulation by ACTH.

This technique could be useful for many studies concerning adrenal metabolism, because the same cells can be tested with a stimulant and then an inhibitor, without problems of end-product interference. Column perfusion could also be very useful for studies of kinetics, dose response, and the time-course of a response. Future studies of adrenal physiology will prove column perfusion of isolated rat adrenal cells a versatile and valuable tool.

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