1970

The isolation and effects of steer and rabbit adrenocorticotropic hormone on corticosteroid biosynthesis of the rabbit adrenal cortex

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THE ISOLATION AND EFFECTS OF STEER AND RABBIT ADRENOCORTICOTROPIC HORMONE ON CORTICOSTEROID BIOSYNTHESIS OF THE RABBIT ADRENAL CORTEX

By

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B.A., University of Washington, 1967

Presented in partial fulfillment of the requirements for the degree of

Master of Science

UNIVERSITY OF MONTANA

1970

Approved by:

Chairman, Board of Examiners

Dean, Graduate School

Sept 15, 1970
Date

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. H. Richard Fevold, Associate Professor of Chemistry, University of Montana, for his advice, guidance, and encouragement throughout the course of this research.

Appreciation is also due Dr. Galen P. Mell, Assistant Professor of Chemistry, and Dr. Walter L. Koostra, Assistant Professor of Microbiology for their assistance in writing this thesis.

A major portion of this investigation was supported by research grants to Dr. Fevold from the National Institute of Arthritis and Metabolic Diseases No. 2R01 AM08386.

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INTRODUCTION

Adrenal Cortex: Historical Introduction

Understanding of the function of the adrenal gland, the existence of which had been reported as early as 1594, began in 1855 when a British surgeon named Addison described a disease which now bears his name (Thorn, 1968). Aroused by Addison's work, Brown-Sequard, in 1896, undertook to reproduce this syndrome in rabbits, dogs, guinea pigs, and rats by extirpation of the adrenal glands. Due to the uniformly fatal results of all these bilateral adrenalectomies, he postulated that the adrenals were necessary for life. It is probable that death was most often due to operational shock, rather than to lack of the adrenal secretions (Dorfman, 1962). However, Beidl and other workers later confirmed Brown-Sequard's conclusions by more adequate and reliable techniques, proving that the cortex, not the medulla, was necessary for life (Dorfman, 1962).

Adrenocorticotrophin, or ACTH, has been studied during the past forty-five years beginning with the work of Smith (1925, 1930), who described the degeneration of the adrenals, especially the cortex, after hypophysectomy, and their subsequent regeneration after replacement therapy with fresh, ground pituitary tissue. The first homogeneous active fractions of ACTH were not obtained until 1942, when Li, Simpson, and Evans (1942) and Sayers, White, and Long (1942) isolated a fraction

with molecular weight of approximately 20,000 from sheep and pig pituitaries.

The classical in vivo studies of the controlling factors involved in the process of corticosteroidogenesis were carried out by Hechter et al. at the Worchester Foundation during the early 1950's. These studies, which consisted of perfusion of bovine adrenal glands, showed that adrenocorticotrophin could trigger off mechanisms necessary for adrenal steroid biosynthesis (Hechter et al., 1953; Hechter, 1954; Macchi and Hechter, 1954).

ACTH: Structure and Isolation

The primary structure of ACTH from four species has been reported during the past fifteen years, beginning with sheep: or α_s-ACTH (Li et al., 1955), porcine: or α_p-ACTH (Shepherd et al., 1956), bovine: or α_b-ACTH (Engel and Fredricks, 1959), and the partial structure of human: or α_h-ACTH (Lee, Lerner, and Buettner-Janusch, 1960), as illustrated in Figure 1. All structures thus far determined consist of 39 amino acids, and species variations occur in the region of the molecule (positions 25-33) which appears to be nonessential for biological activity (Dixon, 1964). The "active" fragment (positions 1-24) is identical in all species investigated (Dixon, 1964). The calculated molecular weight for porcine ACTH is 4567 (Brown et al., 1956).

Isolation procedures are aided by the small molecular weight of the ACTH molecule which allows the use of some techniques not applicable to larger proteins. This molecule has never been "denatured", as it appears that any change in its properties is accompanied by a change in

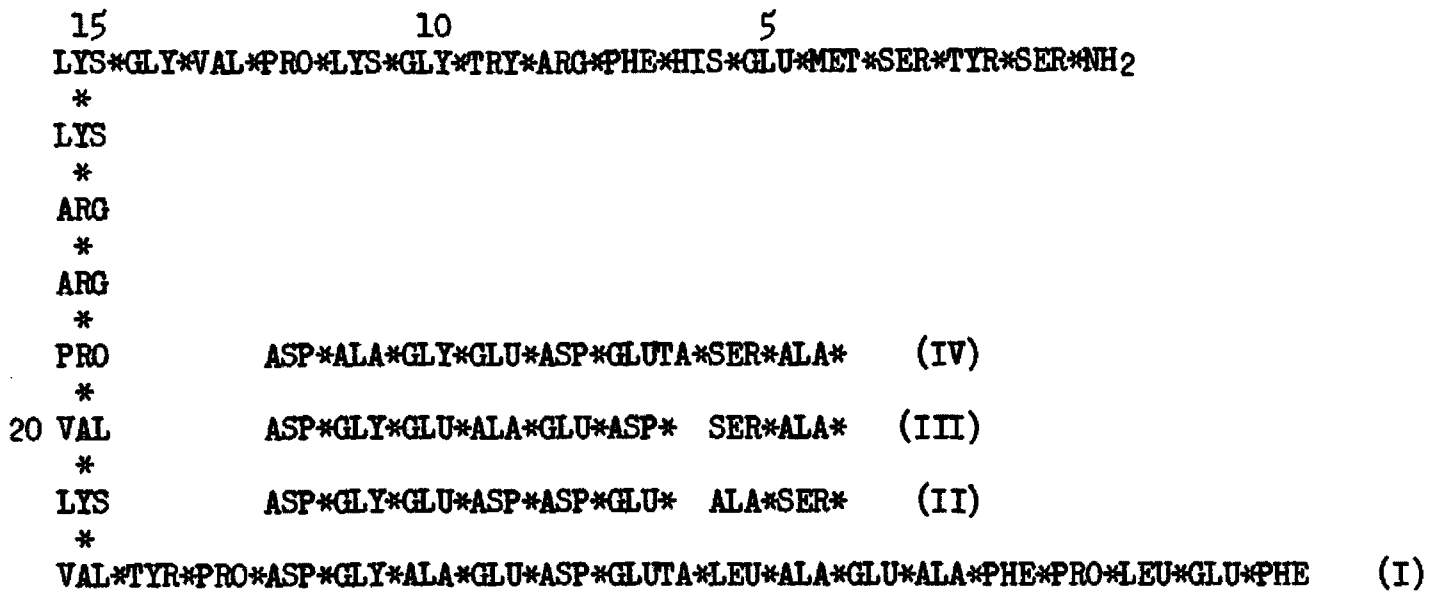

FIGURE 1. Amino acid sequences of pig (I), sheep (II), beef (III), and human (IV) corticotropin (Hofmann, 1962).

its covalent structure (Dixon, 1964). Its low molecular weight also explains its unusual solubility, a feature which, along with those cited above, allows the use of acid extraction procedures developed by Lyons (1937) using 0.2N HCl in 80% acetone and those of Payne, Raben, and Astwood (1950) using hot glacial acetic acid. Dialysis as a method of purification is difficult since ACTH can pass through dialysis membranes under normal conditions. Li (1958) was able to dialyze against an alkaline solution (ammonia) to remove the salts, but this procedure was difficult and tended to slightly alter the molecular structure.

ACTH: Biological Action

Mechanistic information about the action of ACTH is limited almost exclusively to steroidogenesis. There exists, however, a host of other real or apparent actions of ACTH, which are difficult or impossible to study *in vitro*. One such effect is that of ascorbic acid release from the adrenals, which thus far has not been linked to steroidogenesis, but is the basis for one of the most successful bioassay procedures for ACTH activity (Sayers, 1948). Sugar (specifically D-xylose) entry into the adrenal cell is increased by ACTH *in vivo*, but cannot be studied *in vitro* because it is not excluded under these conditions (Golden, 1961). Difficulties arise in the utilization of *in vitro* techniques due to the following changes in structure and function: potassium is lost from the cell while sodium concentration increases (Golden, 1961); intracellular enzymes escape into the medium (Shönbaum, 1959); medullary tissue is destroyed, liberating catchecholamines and/or proteolytic enzymes into the system (Hechter, 1954). Therefore it cannot be

determined how many or in what way the cells are destroyed or altered in an *in vitro* system.

Labile proteins have been postulated to play a role in controlling steroidogenesis and hence their synthesis is another effect under the control of ACTH. That ACTH increases adrenal protein synthesis has been demonstrated by Farese (1967) and Streeto and Reddy (1967). Grower and Bransome (1968) have suggested that the synthesis of some proteins and not others is favored by ACTH, as they demonstrated on acrylamide gel-electrophoresis a selective increase in certain soluble proteins. Farese and Reddy (1963) were the first to deal with this problem, showing that some factor in the 105,000 x g supernatant was rate limiting for *in vitro* adrenal protein synthesis, and that this factor was increased by ACTH administration. Farese (1966) went on to show that continued ACTH treatment led to increased protein synthetic activity of the adrenal polysomes. Farese (1967) suggested that stimulatory effects were due to dissociation of nucleoprotein complexes with consequent enhancement of template activity. He went on to suggest the following possibilities for the proposed unstable regulatory proteins: a) RNA polymerase itself, b) proteins which may influence RNA polymerase, c) proteins which may activate chromatin templates, and d) polycationic substances.

Related to the problem of protein synthesis is that of the relative change in the amount and activity of the RNA present in the cells before and after stimulation. Net increases in adrenal RNA occur soon after ACTH administration (Fiala, Sproul, and Fiala, 1956), and increased incorporation of precursor into RNA can be observed *in vivo* shortly after

ACTH stimulation (Bransome and Chargraff, 1964). Although Imrie and Hutchinson (1965) suggested that this RNA increase is due to decreasing RNA degradation, Farese (1967) demonstrated two phases in the stimulatory effect of ACTH on incorporation of nucleoside triphosphates into RNA, the first occurring during the early hours of stimulation and the second after four hours stimulation. He could not, however, determine if both were under the control of the same mechanism.

The relationships of ACTH to the NADPH level or location in the cell, of ACTH to cyclic 3'5'-AMP, and of cyclic 3'5'-AMP to NADPH have received considerable attention in the past decade. That NADPH is necessary for steroidogenesis was demonstrated by Sweat and Liscomb (1955) in their work on the formation of adrenal corticosteroids. Using adrenal homogenates, McKerns (1964) studied the activation of glucose-6-phosphate dehydrogenase by ACTH. He stressed the importance of glucose-6-phosphate as a substrate for this enzyme and the importance of this enzyme activity in regulating cellular NADPH concentration. He postulated that ACTH exerts its main influence on steroidogenesis at this point. His work has been difficult to reproduce and there have been reports that question the importance of NADPH concentrations as a significant controlling factor (Bransome, 1968). Haynes and Berthet (1957) put forth a theory that ACTH stimulated α-glucan phosphorylase activity in bovine adrenal slices, which increased the availability of glucose-6-phosphate as a substrate for dehydrogenation by glucose-6-phosphate dehydrogenase, which in turn would increase the level of reduced NADPH in the cell. This theory, too, has been challenged by the studies of Harding and Nelson (1966) and Cammer and Estabrook (1966), who favor

the concept of reversed electron transfer involving a linkage of the P-450 chain to the respiratory chain through transhydrogenation (for a good review, see Bransome, 1968).

Corticosteroid Biosynthesis

It is generally believed that the corticosteroids are derived from cholesterol via pregnenolone and progesterone.[1] Tchen (1967) has summarized the cholesterol sidechain cleavage diagramatically as shown in Figure 2. This cleavage involves two hydroxylations and a 'desmolase' reaction in the mitochondrial fraction. Both ACTH and cyclic 3'5'-AMP appear to have their principal effect specifically at the 'desmolase' step (Stone and Hechter, 1954; Karaboyas and Koritz, 1965).

Samuels and Uchikawa (1967) have shown that the major pathways to the glucocorticosteroids from pregnenolone are through progesterone in most species, and that in all species, some progesterone is hydroxylated at C-21 to form 11-deoxycorticosterone (see Figure 3). The step from pregnenolone to progesterone is one of converting a Δ^5-3β-hydroxysteroid

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[1]The following common names are used: pregnenolone, 3β-hydroxy-5-pregnene-20-one; pregnenolone acetate, 20-oxo-5-pregnen-3β-yl acetate; 17α-hydroxypregnenolone, 3β,17α,21-trihydroxy-5-pregnene-20-one; 17α-hydroxypregnenolone acetate, 3β,17α-dihydroxy-5-pregnene-20-one 3β-monoacetate; progesterone, 4-pregnene-3,20-dione; corticosterone, 11β, 21-dihydroxy-4-pregnene-3,20-dione; corticosterone-21-acetate, 11β,21-dihydroxy-4-pregnene-3,20-dione 21-monoacetate; cortisol, 11β,17α21-trihydroxy-4-pregnene-3,20-dione; cortisol-21-acetate, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione 21-monoacetate; 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 11-deoxycorticosterone-acetate, 3,20-dioxo-4-pregnene-21-yl-acetate; 11-deoxycortisol, 17α,21-dihydroxy-4-pregnene-3,20-dione; 11-deoxycortisol-21-acetate, 17α,21-dihydroxy-4-pregnene-3,20-dione; 11-dehydrocorticosterone, 21-hydroxy-4-pregnene-3,11,20-trione; cortisone, 17α,21-dihydroxy-4-pregnene-3,11,20-trione.

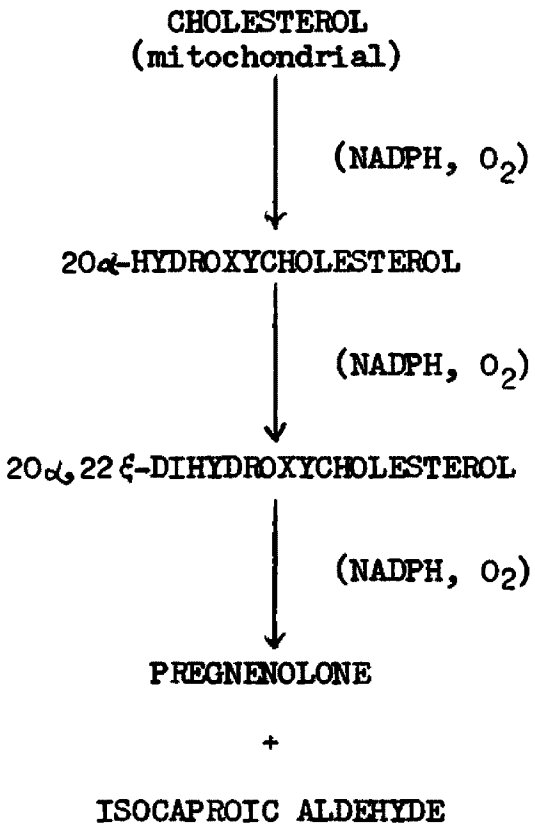

FIGURE 2. Mechanism of cholesterol side-chain cleavage by adrenal cortex (Bransome, 1968).

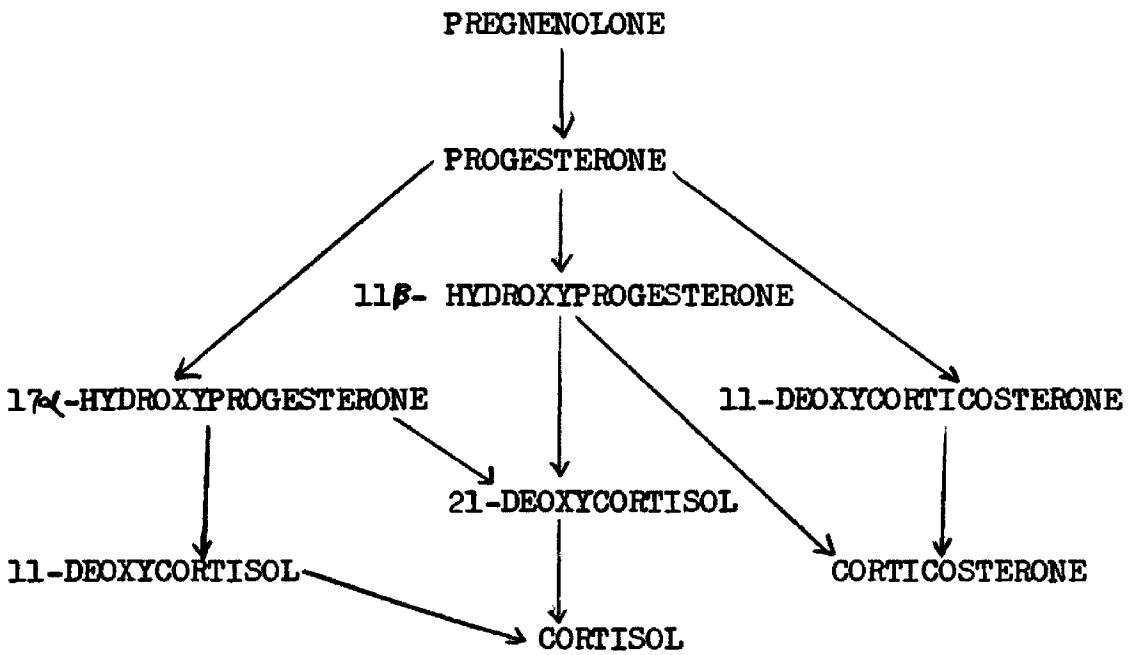

FIGURE 3. Biosynthesis of cortisol and corticosterone (Dorfman and Ungar, 1965).

to a Δ^4-3-ketosteroid and involves a 3β-ol-dehydrogenase and a Δ^5-3-ketosteroid isomerase, both of which are found in the 'microsomal' fraction prepared from adrenal endoplasmic reticulum (Samuels et al., 1951). In the rabbit and most rodents, this is the major pathway to corticosterone. However, in cortisol producing species, the majority of the progesterone undergoes 17α-hydroxylation in the 'microsomes' first, then 21-hydroxylation to 11-deoxycortisol, and finally 11β-hydroxylation to cortisol.

Using perfusion studies, Eichhorn and Hechter (1958) were able to show that 11β-hydroxyprogesterone can be converted to cortisol, that 17α-hydroxyprogesterone forms only cortisol as the 17-hydroxy group is not removed, and that 11-deoxycorticosterone is converted only to corticosterone. In an earlier study Eichhorn and Hechter (1957) were unable to demonstrate a conversion of corticosterone to cortisol.

Carstenson, Oertel, and Eik-Nes (1959) discovered 17α-hydroxypregnenolone in canine adrenal vein blood. This led to the elucidation of another pathway from pregnenolone to cortisol via 17α-hydroxypregnenolone (see Figure 4). 17α-Hydroxypregnenolone is converted to 17α-hydroxyprogesterone, which can be hydroxylated at C-21, then converted to cortisol by 11β-hydroxylation. Hydroxylation at C-21 of 17α-hydroxypregnenolone gives 3β,17α,21-trihydroxypregn-5-en-20-one which can form either 11-deoxycortisol (Berliner, Cazes, and Nabors, 1962) or the Δ^5-3β-hydroxy analog of cortisol, which can give cortisol by oxidation at C-3 (Dorfman and Ungar, 1965).

Figure 5 illustrates the pathways to corticosterone from pregnenolone. Berliner, Cazes, and Nabors (1962) demonstrated the pathway from pregnenolone to 11-deoxycorticosterone via 21-hydroxypregnenolone.

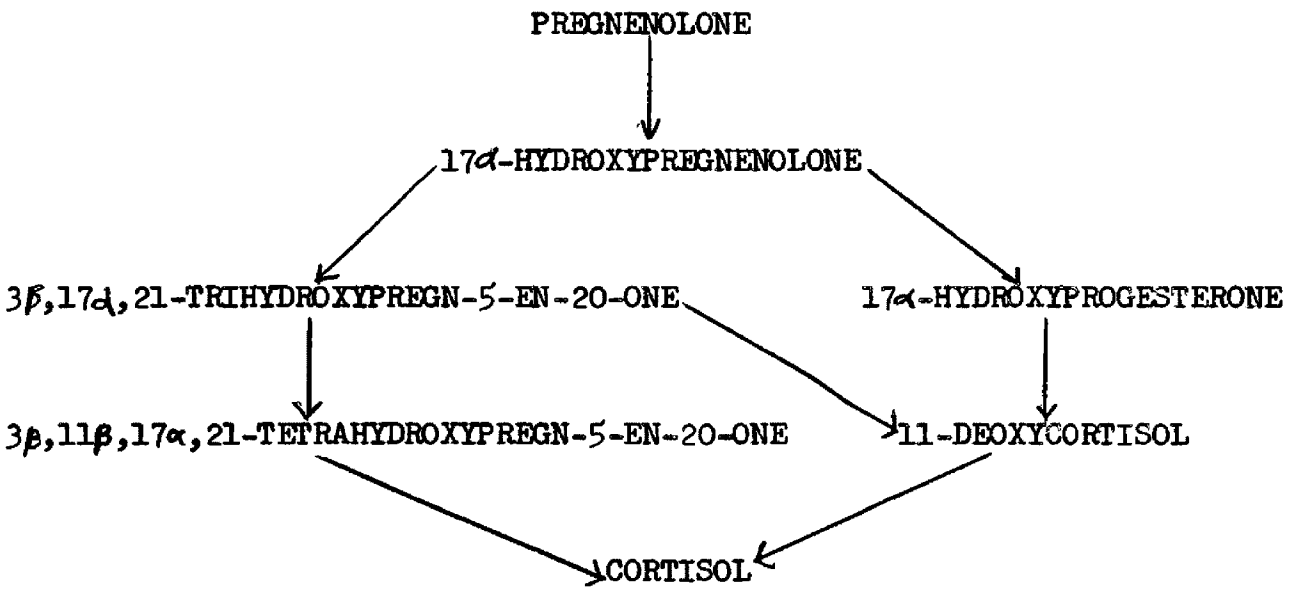

FIGURE 4. Biosynthesis of cortisol through 17α-hydroxypregnenolone (Dorfman and Ungar, 1965.

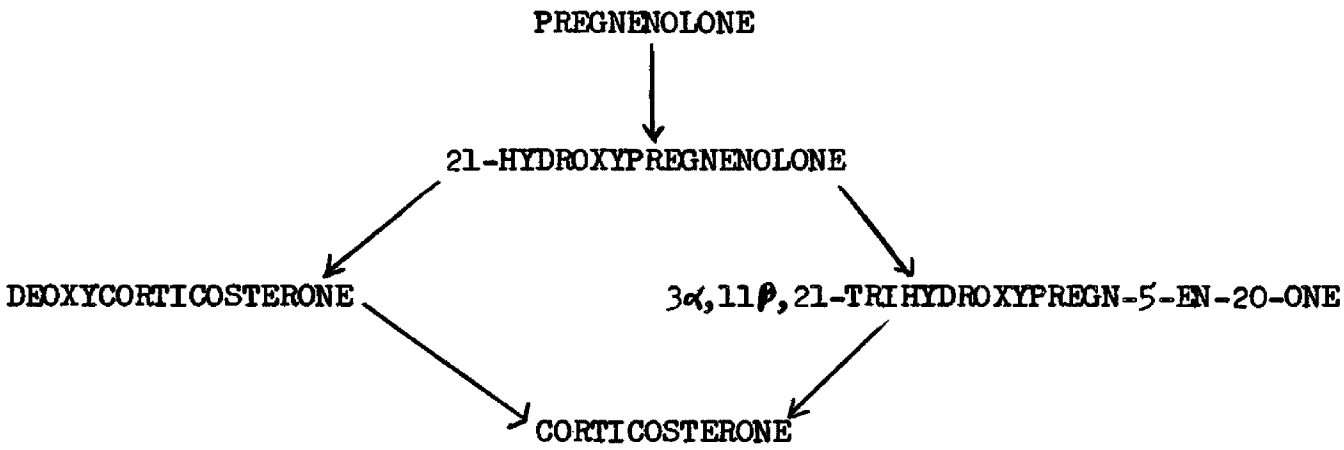

FIGURE 5. Biosynthesis of corticosterone through 21-hydroxy-pregnenolone (Dorfman and Ungar, 1965).

21-Hydroxypregnenolone could also be 11-hydroxylated to 3β,11β,21-trihydroxypregn-5-en-20-one, which would form corticosterone via the action of Δ^5-3β-ol-dehydrogenase (Dorfman and Ungar, 1965).

Adrenal Hydroxylases

The formation of the corticosteroids from pregnenolone and progesterone is the result of hydroxylation reactions at the 11β, 17α, and 21 positions of the steroid molecule. These hydroxylations are aerobic reactions catalyzed by enzymes designated as 'mixed-function oxidases' which activate molecular oxygen and cause the incorporation of one atom of oxygen into the substrate. The other oxygen atom accepts the hydrogen taken from the substrate and, along with a proton, becomes a water molecule (Dorfman and Ungar, 1965). The oxygen atom of the hydroxyl group is stable to exchange and no isotope appears in the product when the reactions are carried out in D_2O or H_2O^{18} (Taladay, 1965).

Some interesting observations regarding steroid hydroxylases are as follows. The 17α-hydroxylase activity has not been separated from the 21-hydroxylase activity, both of which are located in the microsomal fraction of the adrenal cell (Ryan and Engel, 1957). 11-Deoxycorticosterone was not 17-hydroxylated by a preparation of 17α-hydroxylase, whereas pregnenolone was transformed to 11-deoxycortisol (Dorfman and Ungar, 1965). Progesterone and 17α-hydroxyprogesterone were converted by adrenal homogenates to 11-deoxycorticosterone and 11-deoxycortisol, respectively, with equal facility, while 11β-hydroxyprogesterone and 21-deoxycortisol could be hydroxylated also (Dorfman and Ungar, 1965). Hyano (1962) explains these reactions as follows: "The enolization of

17α-hydroxyprogesterone at carbons 20,21 with enzymatic hydroxylation at C-21 is straight forward. In the case of progesterone, enolization is possible in two directions, between carbons 17,20 (A) and between 20,21 (B). Both are favored states: (A), kinetically and (B), thermodynamically. Thus, at specified enzyme loci 17α- and 21-hydroxylations of appropriate enol forms may proceed. With 11-deoxycorticosterone on the other hand, the 20,21 enol is the form both thermodynamically and kinetically favored, allowing only a very low contribution of the 17,20 form. Thus, theoretically, 17α-hydroxylation of this structure is essentially impossible. This is indeed observed in incubations with this substance, where no more than trace quantities of 11-deoxycortisol have ever been noted."

The 11β-hydroxylase system, which is found in the mitochondria, is much more thoroughly studied than either the 17α- or 21-hydroxylase systems. On the basis of kinetic data showing rates of hydroxylation proportional to substrate concentration, Sharma, Forcheilli, and Dorfman (1962) suggested that 11-deoxycorticosterone and 11-deoxycortisol are hydroxylated by the same reaction sites on the 11β-hydroxylase and are therefore competitive. Corticosterone and cortisol were not found to inhibit the hydroxylation of 11-deoxycortisol and 11-deoxycorticosterone.

The question of hydroxylase specificity arises due to the fact that hydroxylation reactions of adrenal steroids are supported by NADPH, involving a transhydrogenation and reverse electron flow along a coenzymatic chain containing cytochrome P-450. Specificity of the hydroxylases may be due to multiple forms of cytochrome P-450. The great precedent for genetic differences in cytochromes lends support to this theory (Bransome, 1968).

Corticosteroidogenesis: The Rabbit

The secretory products of the adrenal cortex have been studied both *in vivo* and *in vitro* for a variety of diverse species. Bush (1953) found that the major steroid present in the adrenal vein blood was either cortisol, corticosterone, or a mixture of both. He further suggested that the ratio of adrenal steroids remained constant after ACTH administration. Pigs, sheep, and monkeys have been reported to secrete primarily cortisol; rabbits and rats secrete primarily corticosterone; while cows, ferrets, cats, and dogs secrete mixtures of the two (Bush, 1953).

After observing the effects of ACTH and corticosteroids on the ribonucleic acid (RNA) of lymphoid tissues of the rabbit, Kass *et al.* (1954) suggested that this ratio of cortisol to corticosterone may not be constant. It was found that ACTH, cortisone, and cortisol caused a change in the weight of RNA in the lymphoid tissue, whereas corticosterone, even in relatively large doses caused no change. This suggested that the prolonged administration of ACTH must somehow alter the adrenal steroid secretion from primarily corticosterone to an increased level of cortisol. Kass *et al.* (1954) suggested that this increase was due to the rate of hydroxylation of progesterone at C-17 and C-21, probably due to the absence of inhibition of the C-17 hydroxylating system. Krum and Glenn (1965) repeated the work of Kass and coworkers, and postulated a scheme for the biosynthetic pathway in rabbit adrenals (see Figure 6).

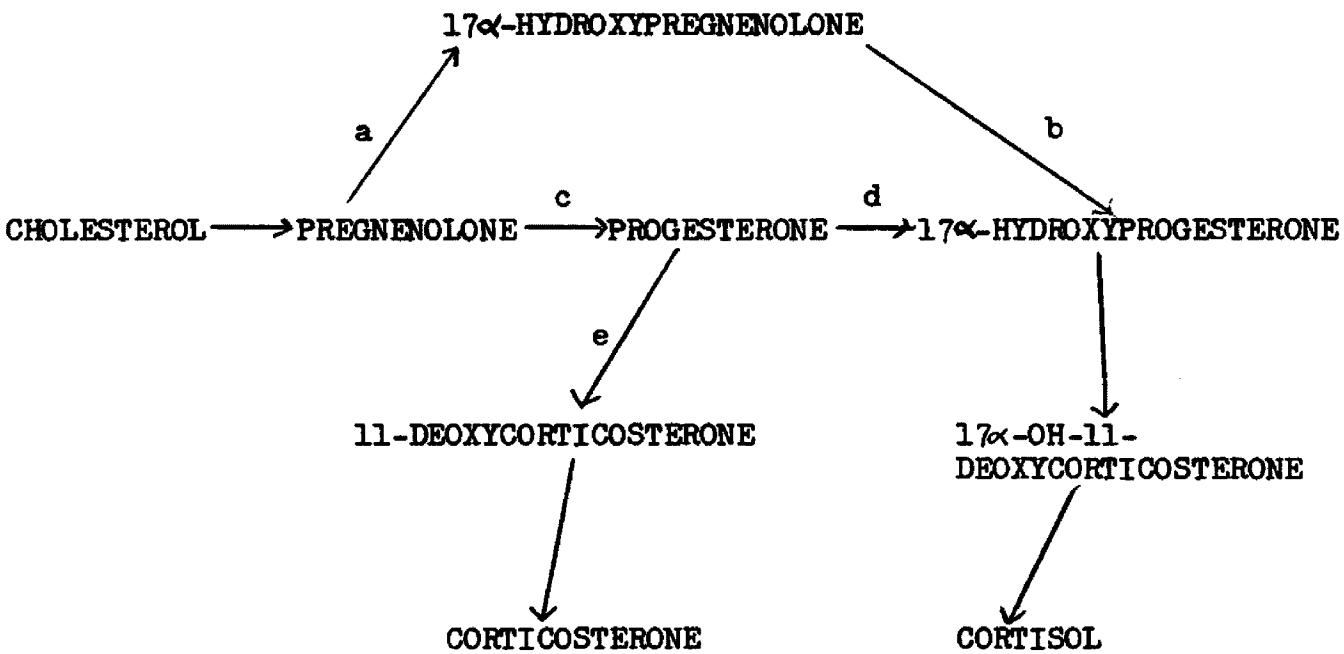

FIGURE 6. Steroidogenesis in the rabbit adrenal cortex (Krum and Glenn, 1965).

Kass and coworkers (1954) had proposed an increase in step d (Figure 6) with a possible decrease in e; whereas Krum and Glenn (1965) proposed an increase in step a and/or a reduction in step c. Yudaev and Morozova (1965), incubating rabbit adrenal slices from animals stimulated 24 days with ACTH, found an increase in cortisol with a decrease in corticosterone production from pregnenolone. They suggested that this was due to a partial inhibition of the 21-hydroxylase enzyme.

Fevold (1967) suggested that the loss of 21-hydroxylase activity per gram adrenal tissue reported by Yudaev and Morozova (1965) could be explained by the fact that the weight of adrenal tissue from the ACTH stimulated animals was twice that of the controls. In his work, Fevold (1967) found little evidence supporting an inhibition of the 21-hydroxylast system in stimulated tissue.

Fevold (1967) determined that the 17α-hydroxylase enzyme activity has a relative specificity for Δ^5-pregnenolone over progesterone. He also reported that a relatively large excess of progesterone could inhibit the more efficient conversion of endogenous Δ^5-pregnenolone to cortisol via the 17α-hydroxylase system. It is also interesting to note that the cortisol to corticosterone ratio is dependent upon the substrate concentration (pregnenolone or progesterone) such that at high concentrations the cortisol to corticosterone ratio may be quite small, whereas at low concentrations it may approach one, or even surpass it, giving credence to the early findings of Kass et al. (1954), who reported cortisol to corticosterone values greater than one (Fevold, 1967).

Another important step along this line was taken when Fevold (1968) showed that this 17α-hydroxylase activity could be induced by stimulation

of rabbits with 0.4 mg synthetic β^{1-24}-corticotropin per day for two days. Although the 17α-hydroxylase activity reported was less than that obtained after stimulation with natural (porcine) ACTH, it showed that this effect was due to the ACTH molecule and not to some other impurity present in the natural preparation. This lesser activity may have been due to a decreased stability of the tetracosopeptide when injected intramuscularly as reported by Landon (1964).

CHAPTER II

STATEMENT OF THE PROBLEM

Apparent alterations in the steroidogenic pathways of the rabbit adrenal cortex as a result of chronic stimulation by porcine adrenocorticotropin for extended periods of time may reflect the structure of the actual ACTH molecule injected into the animal. All previous experiments on this problem have used ACTH from primarily cortisol producing species, the exception being the studies involving synthetic β^{1-24}-corticotropin, which itself is structured after porcine ACTH. It was of interest, therefore, to attempt to duplicate this action using ACTH from a primarily corticosterone-producing species to determine whether the type of ACTH used affected the end result of this stimulation. The focus of this research, then, was to isolate ACTH from a corticosterone-producing species and to observe its effect on the biogenic pathways of the rabbit adrenal cortex. Since the rabbit is a corticosterone-producing species, and also the subject of the adrenal stimulation studies, it was selected as the ideal source for the ACTH.

It was also desired to make some comparison of this ACTH from a corticosterone-producing species with ACTH from a cortisol-producing species. For lack of the proper equipment, some comparison short of the primary amino acid sequence had to be used. Disc gel electrophoresis on polyacrylamide gel was selected for this purpose.

CHAPTER III

EXPERIMENTAL METHODS AND MATERIALS

Animals and Pituitary Tissue

1. Pituitaries. Raw, frozen steer and rabbit pituitaries were purchased commercially from Pel-Freez, Inc.
2. Rats. Male albino rats of the Sprague-Dawley strain weighing approximately 200 g, which were used for the ACTH assays, were ordered from Rush Laboratories, Inc.
3. Rabbits. Male New Zealand white rabbits weighing 2.3 to 3.2 kg were injected with ACTH preparations, and their adrenal glands were subsequently used for the in vitro incubations.

Note: All animals were allowed to adjust to their new environment in the basement animal rooms of the Health Science Building of the University of Montana for a period of at least three days before use in any of the following experiments.

Chemicals

1. Radioactive Materials
   a. Pregnenolone-4-^{14}C, CFA 271, Batch 5, Nuclear-Chicago.
   b. Corticosterone-1,2-^{3}H, lot #925-20-120, Tracer Lab.
   c. Cortisol-1,2-^{3}H, TRK 133, Batch 9, Nuclear-Chicago.
   d. 11-Deoxycorticosterone-1,2-^{3}H, lot #925-22-44, Tracer Lab.
   e. 17α-Hydroxyprogesterone-1,2-^{3}H, Mann Laboratories.

   Note: All were chromatographically purified before use.

2. Cofactors and Additives

a. Adenosine triphosphate (ATP) 99% pure, control No. 6211, Nutritional Biochemical Corp.

b. Nicotinamide, Metheson Coleman and Bell Company.

c. Sodium fumarate (Fu 15684), C. F. Boehringer and Soehne Gmbh, Mannhein Co.

d. Triphosphopyridine nucleotide (NADP), lot No. 16-B-7002, and Diphosphopyridine nucleotide (NAD), lot No. 15-B-7260, Sigma Chemical Co.

3. Buffers

a. Krebs-Ringer bicarbonate buffer and Krebs-Ringer phosphate buffer were prepared according to Umbreit (1957).

b. Electrophoresis buffers were prepared according to the instructions for the Polyanalyst Disc Electrophoresis Apparatus supplied by Buchler Instruments, Inc.

c. Ammonium Acetate buffers used with CM-cellulose column.

4. Standard Steroid Solutions

Steroid solutions were prepared by dissolving weighed quantities of crystalline steroids in redistilled absolute ethanol. Concentrations of Δ^4-3-keto steroids were determined by their UV absorbence at 240 nm.

5. Solvents

All solvents that were not spectral grade were redistilled before use.

6. Chromatography Paper

Whatman No. 1 filter paper (46 x 57 cm) was cut with 2 and 3 cm lanes for descending chromatography.

7. Scintillation Fluid

4 Grams PPO (2,5-diphenyloxazole) and 50 mg POPOP [2-P-phenylene bis(5-phenyloxazole)] were dissolved in 1 l spectral quality toluene.

8. Gases

Water-pumped nitrogen and a mixture of carbon dioxide:oxygen (5%-95%) were supplied by Industrial Air Products, Inc.

9. ACTH

Purified porcine ACTH (lot No. 7275) was donated by Dr. J. W. Hinman of the Upjohn Company.

10. Miscellaneous Chemicals

a. Carboxymethyl cellulose--Eastman Organic Chemicals #7796.

b. Blue Tetrazolium Reagent--Calbiochem #203592.

c. Pyridine, redistilled under vacuum and stored in desicator under vacuum--Mallinckrodt Chemical Works #7180.

d. Tetramethyl ammonium hydroxide, 10% in H_2O--Eastman Organic Chemicals.

e. Acetic Anhydride, redistilled under vacuum and stored under vacuum--Mallinckrodt Chemical Works #2420.

f. Formamide, redistilled under vacuum--Matheson, Coleman, and Bell #2749.

g. Glucose-6-phosphate dehydrogenase, type VI--Sigma Chemical Co.

Preparation of ACTH

1) Acetone-Acid Powder

The procedure for the isolation and purification of ACTH from steer and rabbit pituitaries was adapted from the previous work of C. H. Li and his associates (Li et al., 1955a; Li, 1956; Pickering, 1963; Li and Burk, 1964). Whole frozen pituitaries were homogenized in a Waring blender with 83% acetone in hydrochloric acid (0.5 l H_2O:4.1 l acetone: 0.1 l HCl) at 4°C for two minutes, using a volume of 4.1 l of this solution per kilogram of frozen pituitaries. After stirring the suspension in an ice bath for one hour, the mixture was filtered. The residue was re-extracted with 80% aqueous-acetone (2 l per kg original material) and mixed for one hour. After filtration, this filtrate, combined with the previous one, was poured into a large volume of cold

acetone (30 l per kg original material) and allowed to stand overnight. After approximately 12 hours the precipitate was collected by decanting the supernatant fluid, washed with cold acetone, and dried under vacuum. Henceforth, this fraction will be referred to as AAP for acid-acetone powder.

2) Fraction D

The AAP was dissolved in deionized water (47 ml/g) and the pH was adjusted to 3.0. The precipitate formed at 4°C following addition of saturated NaCl solution (30 ml/g AAP) was collected by centrifugation and discarded. Solid NaCl was added to the supernatant until saturated, causing the formation of a precipitate, which was also collected by centrifugation. This precipitate was dissolved in deionized H_2O (5 ml/g AAP) and dialyzed against distilled H_2O at 4°C for 48 hours. This fraction will henceforth be referred to as Fraction D.

3) Final ACTH Fraction

A 2 g sample of Fraction D was applied to a carboxymethyl cellulose chromatography column (60 x 1 cm) previously equilibrated with 0.01 M ammonium acetate buffer, pH 4.6 (Li and Burk, 1964). Four milliliter fractions were collected using a Buchler Fractomat. Alternate tubes were read at 278 nm using a Gilford spectrophotometer to verify the absorbence recorded by the liquid flow analyzer. The first buffer, 0.01 M ammonium acetate buffer (pH 4.6), was employed until three to four holdup volumes had been collected. A gradient with respect to pH and concentration was then started by introducing 0.1 M ammonium acetate buffer (pH 6.7) through a 500 ml mixing flask (500 ml Erlenmyer flask equipped with

a magnetic spin bar) containing the starting buffer (Pickering et al., 1963). After several peaks had been eluted and the absorbancy of the eluate approached the baseline value, the gradient was increased by substituting 0.2 M ammonium acetate (pH 6.7) as the solution flowing into the mixing flask.

The eluates corresponding to the various protein peaks were lyophilized several times to remove the ammonium acetate and the resultant dried fractions were assayed for biological activity.

4) ACTH Assay

The lyophilized protein fractions of the steer ACTH preparation from the CM-cellulose column were assayed in our laboratory according to the procedure of Saffron and Schally (1955; 1965). The rabbit ACTH preparation was assayed by G. W. Camiener and P. N. Tree of the Upjohn Company using a method of C. H. Li as modified by G. W. Camiener, using Acthar (Armour) as the standard. These men also assayed the procine ACTH (Upjohn #7275) that was used as the standard for the steer ACTH assay.

The steer ACTH assay required the adrenal glands of six to eight male albino rats (body weight approximately 200 g). The animals were sacrificed by decapitation to avoid the introduction of any drugs that might have affected the assay. Caution had to be taken to avoid excitation which could have led to the secretion of endogenous ACTH introducing a questionable effect on the results. The adrenals were immediately removed, dissected free of all fat and connective tissue, weighed, placed in each of eight 25 ml glass-stoppered Erlenmyer incubation flasks containing 1.5 ml Krebs-Ringer bicarbonate buffer

(ph 7.35), and placed on ice. Each flask was gassed one minute with bottled 95% O_2-5% CO_2 and preincubated in a shaking water bath at 38°C. After one hour, each flask was removed from the bath, the buffer withdrawn with a transfer pipette, 1.4 ml fresh buffer added, and ACTH solution equivalent to 0.3 or 0.9 mg of protein added. The flasks were again gassed with the O_2-CO_2 mixture and incubated in the shaking water bath for two hours. At this time, 1 ml of the incubation medium (aqueous) was removed and added to 1 ml cold methylene chloride (spectral grade) in a 5 ml glass-stoppered tube. The mixture was shaken thoroughly, and centrifuged (approximately 1500 rpm) to separate the phases. The optical density of the organic phase was read at 240, 250, 255, and 260 nm and the hormonal response determined:

$$\text{Response} = \frac{\text{O.D.}_{240} - \text{O.D.}_{255}}{\text{Adrenal Tissue Wt.}} \times 10$$

O.D._{240} represents the maximum absorption of the corticoid (Δ^4-3-keto-steroid) ring. Spectral scans of adrenal incubation extracts indicate coincidental characteristics with cortisol from 230 to 265 nm and a 240 nm maximum (Saffron and Schally, 1962). Turbidity which develops in the methylene chloride extracts necessitates a correction at the higher wavelength of 255 nm. The response was plotted against the dose on semi-log paper for comparison of the slopes and relative heights. The resultant slopes of these straight lines should be identical for hormones of similar activity. The difference in their heights indicates the relative potency or activity of the hormones being compared (Saffron and Schally, 1965).

5) Electrophoresis of Acth

Disc electrophoresis using 7.5% polyacrylamide gel was carried out on the porcine ACTH (Upjohn), various CM-cellulose fractions of the steer and rabbit ACTH preparations. Materials and methods employed were those accompanying the temperature-regulated Buchler Polyanalyst disc electrophoresis apparatus. The steer, rabbit, and porcine ACTH preparations were run at pH 9.3 on 7.5% polyacrylamide gel. The porcine and rabbit preparations were also run on 7.5% polyacrylamide gel at pH 4.6.

Approximately 200 µg of sample were added to each column. Trace amounts of bromphenol blue were used to mark the buffer front in the anionic system, whereas methyl green was used in the cationic system. A current of 1.25 ma per column was applied until the samples had entered the stacking gel after which it was increased to 2.5 ma per column for the remainder of the run (60 minutes). The gels were stained one hour with 1% Amido Schwarz in 7% acetic acid, then destained at 5 ma per tube with 7% acetic acid in the upper and lower buffer chambers. The gels were stored at 4°C in 7% acetic acid.

Rabbit Adrenal Assay

1) Tissue Preparation

Male New Zealand white rabbits weighing 2.3 to 3.2 kg were injected twice daily for two days with approximately 26 U purified porcine ACTH (Upjohn, lot no. 7275) or the equivalent of 26 U steer or 40 U rabbit ACTH, which had been purified by the preceding method. The corticotropins were suspended by means of a sonifier in 5% beeswax in peanut oil

containing 0.5% phenol as a preservative. Control animals were injected with the beeswax-peanut oil vehicle only. Under anesthesis of pentabarbitol injected in an ear vein, the animals were exsanguinated and the adrenals removed, dissected free of fat and connective tissue, and weighed. Adrenal glands of similarly stimulated animals were pooled and homogenized at 0°C in sufficient Krebs-Ringer phosphate buffer to give a tissue concentration of 100 mg per ml.

2) Rabbit Adrenal Incubation

Substrate, consisting of pregnenolone-4-^{14}C (Nuclear Chicago) which had been previously purified by paper chromatography and whose specific activity had been adjusted to 1.0 μCi and 3.3 μmoles per ml with non-radioactive pregnenolone (Sigma), was added in 0.2 ml propylene glycol-ethanol (1:1) to 25 ml incubation flasks. Each flask, therefore, received 0.66 μmoles and 0.2 μCi pregnenolone. The ethanol was allowed to evaporate under nitrogen at 40°C before the addition of two units glucose-6-phosphate dehydrogenase (Sigma Type VI). To this was added 1.0 ml of homogenized adrenal tissue in Krebs-Ringer phosphate buffer (100 mg tissue) and 1.0 ml Krebs-Ringer phosphate buffer containing sufficient quantities of cofactors and additives to give the final two ml incubation volume the following concentrations: 0.4 mM nicotinamide adenine dinucleotide (NAD), 0.4 mM nicotinamide adenine dinucleotide phosphate (NADP), 40.0 mM nicotinamide, 0.4 mM adenosine triphosphate (ATP), 0.1 mM sodium fumarate, 1 mg/ml β-D-glucose, and 1 mg/ml glucose-6-phosphate.

Each flask was gassed with 95% O_2-5% CO_2 for one minute, stoppered, and placed in a constant temperature shaking water bath at 37.5°C.

Incubations were carried out in duplicate or triplicate for periods of zero and three hours. Addition of 5 ml dichloromethane:ethyl acetate (1:1) terminated the incubation. The incubation medium and the organic solvent were mixed thoroughly and stored at -20°C until extracted.

3) Extraction of Steroids

Approximately 0.5 μCi of chromatographically purified ^{3}H-labeled cortisol, corticosterone, 11-deoxycorticosterone, and 17α-hydroxyprogesterone were added to each flask and to separate counting vials in order to estimate the percent recoveries of these compounds.[1] Deionized water (3 ml) was added to the incubation flasks to bring the total aqueous volume to 5 ml. The incubation media were then extracted four times with 10 ml dichloromethane:ethyl acetate (1:1) with the organic phase from each extraction being added to that of the previous extractions. The combined extracts were evaporated under nitrogen at 40°C in preparation for chromatographic separation.

4) Product Isolation: Chromatography and Acetylation

Residues of the combined extracts were initially separated into three major fractions of paper chromatography on 3 cm strips of Whatman No. 1 filter paper in the heptane/formamide system (Zaffaroni, 1953), developed for one hour after the mobil phase had reached the end of the strip. After the heptane had evaporated, the strips were developed in the chloroform/formamide system for 3-4 hours. A standard mixture of 50 μg each of corticosterone, cortisol, and 11-deoxycorticosterone was

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[1]In the case of the steer ACTH incubation, only ^{3}H-labeled cortisol and corticosterone tracers were added.

run on a separate strip with each chromatogram. The chromatograms were dried overnight at room temperature, then scanned to locate the various steroids--the standards for ultraviolet absorbing materials by means of a Haines Scanner (Haines, 1950), and the samples by a gas-flow strip and thin-layer scanner (Stomic Accessories model RSC 363) equipped with a chart recorder. The sample tracings were compared with the standard strips, and the areas corresponding to the known compounds were cut and eluted with 15 ml redistilled absolute ethanol.

Further paper chromatography was callied out using 2 cm strips of Whatman No. 1 filter paper until the various compounds appeared to be separated. Figure 7 illustrates the scheme utilized in the separation of the corticosteroids resulting from the incubation of rabbit and porcine ACTH-stimulated adrenal tissue.

When the separation of the individual corticosteroids appeared to be complete, acetylation was performed. To the previously dried sample residue was added 0.2 ml pyridine:acetic anhydride (4:1 v/v). The tube was then placed in the dark overnight (8-16 hours). The reaction was stopped by the addition of 2 ml redistilled ethanol. The ethanol and pyridine were then evaporated under nitrogen at 40°C. Standard steroid solutions were treated in like fashion to be chromatographed with the acetylated samples. Final chromatograms were run on 2 cm strips of Whatman No. 1 filter paper previously washed five days with deionized water followed by five days with redistilled methanol.

A brief sketch of the separation of each of the three sections of the original chromatogram follows:

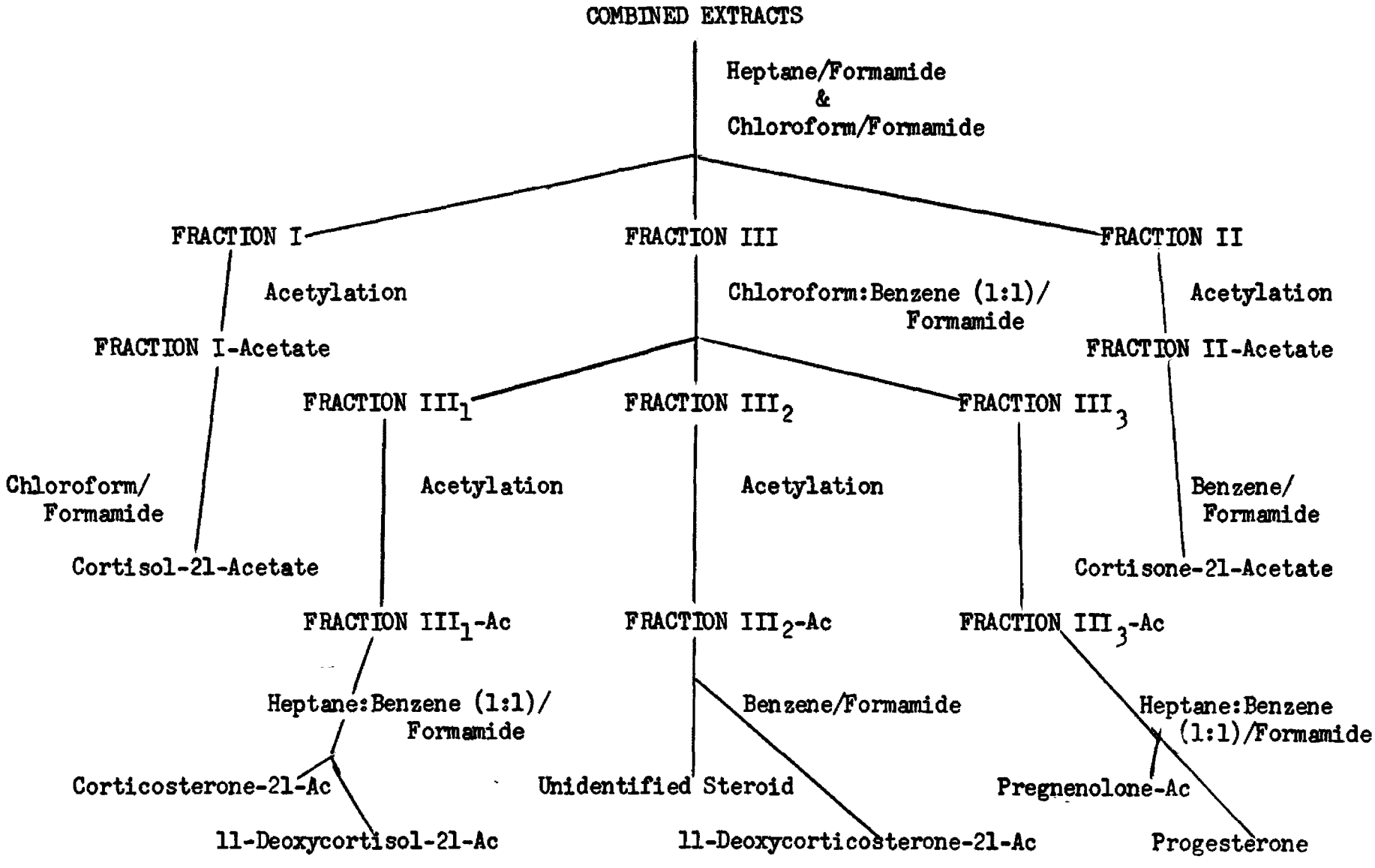

FIGURE 7. Chromatographic separation scheme of adrenal corticosteroids.

FRACTION I (cortisol) was eluted with 15 ml redistilled absolute ethanol, evaporated under nitrogen at 40°C, and acetylated along with a 50 µg standard of cortisol. The acetylated residue was chromatographed in the chloroform/formamide system to the front. The section corresponding to the cortisol-21-acetate section of the standard strip was eluted with 15 ml ethanol, evaporated, and stored in 10 drops ethanol at 4°C for analysis of mass and radioactivity.

FRACTION II (cortisone) was eluted, acetylated, and chromatographed in the benzene/formamide system. The chromatogram was allowed to develop four hours after the mobil phase had reached the end of the strip. The section corresponding to the cortisone-21-acetate section of the standard strip was eluted with ethanol, the solvent evaporated, and stored for analysis as above.

FRACTION III (corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, progesterone, and pregnenolone) was chromatographed in the chloroform:benzene (1:1)/formamide system to the front. The area corresponding to corticosterone and 11-deoxycortisol was eluted, dried, and acetylated. The heptane:benzene (1:1)/formamide system, developed 22 hours after the mobil phase had reached the end of the strip, separated these two compounds. The center of the Fraction III chromatogram was acetylated and chromatographed in the benzene/formamide system to the front. The sections corresponding to 11-deoxycorticosterone-21-acetate, 17α-hydroxypregnenolone-21-acetate, and 11-dehydrocorticosterone-21-acetate were eluted and stored for analysis. The third section of the Fraction III chromatogram containing the least polar compounds, pregnenlone and progesterone, was

developed in the heptane:benzene (1:1)/formamide system after acetylation, and the areas corresponding to progesterone and pregnenolone-3β-acetate were eluted and stored for analysis.

5) Analysis

After all separatory procedures had been completed, the acetylated steroid residue, which had been eluted into a 15 ml centrifuge tube and the solvent evaporated under nitrogen, was stored at 4°C in 10 drops redistilled ethanol, was dried under nitrogen atmosphere at 40°C. One ml redistilled ethanol was added to each tube. The tube was immediately capped, allowed to stand at room temperature for 15 minutes, and subsequently mixed thoroughly for one minute. Two 300 µl aliquots were taken for quantitative analysis of mass and two 100 µl aliquots were taken for analysis of the content of ^{3}H and ^{14}C.

Adrenocortical steroids containing the alpha-ketol side-chain (except the 17α-hydroxy compounds) were quantitatively determined in duplicate by means of the blue tetrazolium (3,3'-dianisole-bis-4,4'-3,4-diphenyl tetrazolium chloride) reaction (Izzo, Keutmann, Burton, 1957; Peŕon, 1962). 17α-Hydroxylated corticosteroids were determined in duplicate by the method of Porter and Silber (1950).

Dual channel liquid scintillation spectrometry was used to determine the ^{14}C and ^{3}H content. Percent recovery values were determined by dividing the amount of tritium present in each sample after extraction and separation by the amount of tritium added to each flask prior to extraction and multiplying this by a factor of ten to correct for the fraction represented in the aliquot analyzed (0.10 ml). The percent conversion of substrate was equivalent to the quotient of the ^{14}C

content (multiplied by a factor of ten to account for aliquot) in the sample divided by the ^{14}C present in the original substrate before incubation. This value was corrected for percent recovery in cases where ^{3}H-tracer compounds had been added prior to extraction. In other cases it represents the relative percent converted.

CHAPTER IV

RESULTS

ACTH Isolation

Data concerning the extraction and purification of ACTH from steer and rabbit pituitaries is presented in Table 1. The curves in Figures 8 and 9 represent the ultraviolet absorbtion (278 nm) of the protein (and other UV-absorbing material) as it was eluted from the carboxymethyl cellulose column. Absorbance at 254 nm was recorded on an Isco liquid flow analyzer and the absorbance of alternate individual fractions (4 ml) were verified at 280 nm with a Gilford spectrophotometer. The dry weights of the resulting proteins from the tubes included under the 'peaks' of the curves are recorded in Table 1.

Weights of the acid-acetone powder, AAP, obtained from the steer and rabbit preparations have been expanded to the values expected from 1 kg of pituitaries based on the recovery from 913 g steer pituitaries and 349.6 g rabbit pituitaries, permitting a comparison of these weights with each other and with previous weights of AAP isolated by this procedure from sheep pituitaries (Li, 1956). This expansion reveals 23.6 g AAP/kg pituitaries for the rabbit preparation as compared with the reported yield of 35 g AAP/kg sheep pituitaries (Li, 1956). Final ACTH preparations resulting from chromatography of 2 g Fraction D on a CM-cellulose column were 110 mg for the steer and 65 mg for the rabbit. Li (1964) reports a yield of 100 mg sheep ACTH from a 2g sample of Fraction D chromatographed by the same procedure.

TABLE 1. Dry weights of ACTH fractions isolated from steer and rabbit pituitaries.

	Steer	Rabbit
Raw pituitary glands	913.0 g	349.6 g
Acid-acetone powder	21.6 g	13.0 g
Acid-acetone powder/kg	23.6 g	28.6 g
Fraction D/20 g AAP	2.12g	3.15g
CM-cellulose fractions/2 g Fraction D		
a) tubes under A*	0.057 g	0.066 g
b) " " B	0.111 g	0.037 g
c) " " C	0.019 g	0.067 g
d) " " D	0.024 g	0.052 g
e) " " E	0.011 g	0.065 g
f) " " F		0.044 g

* Designated on Figures 8 and 9.

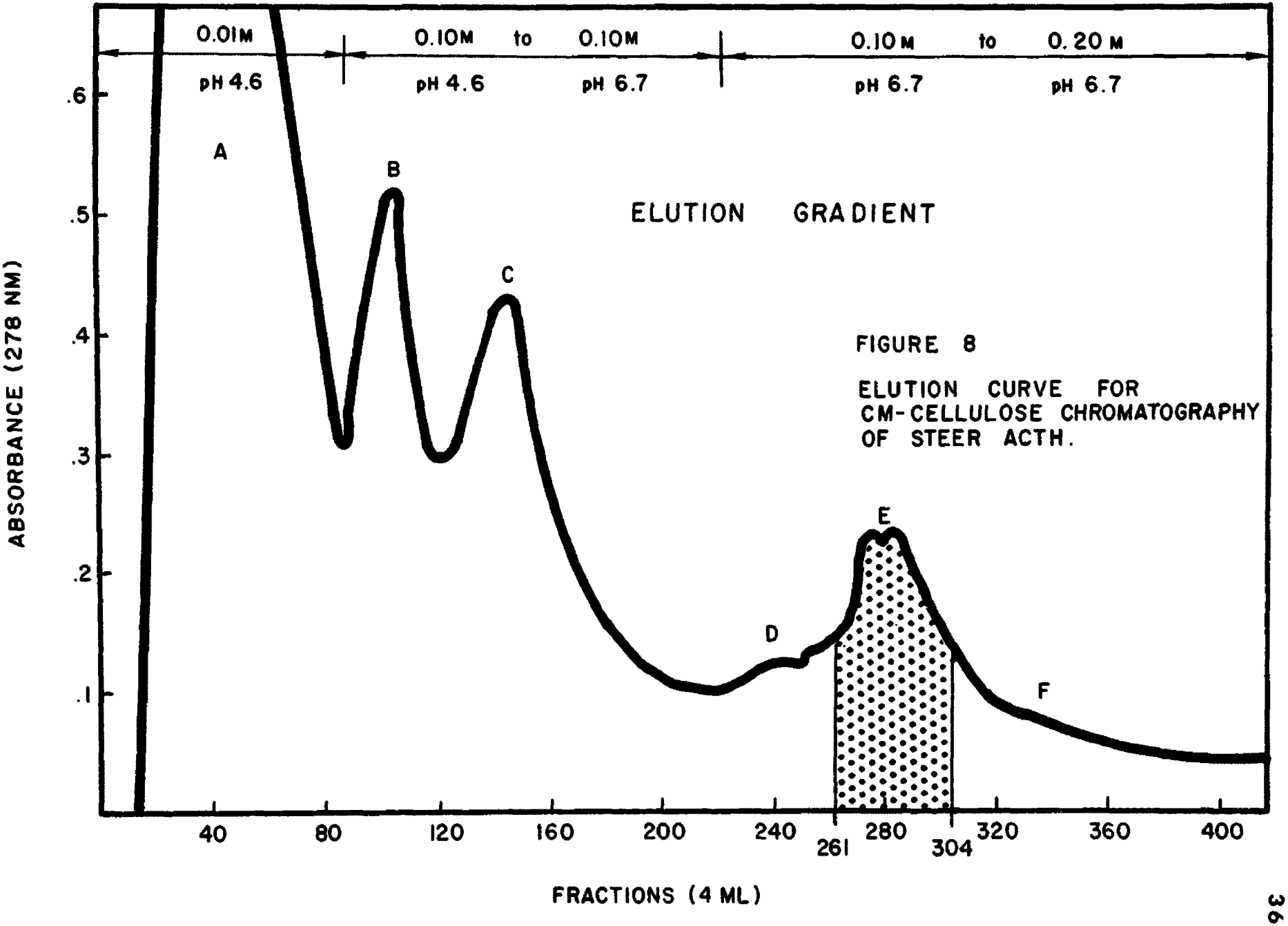

FIGURE 8

ELUTION CURVE FOR CM-CELLULOSE CHROMATOGRAPHY OF STEER ACTH.

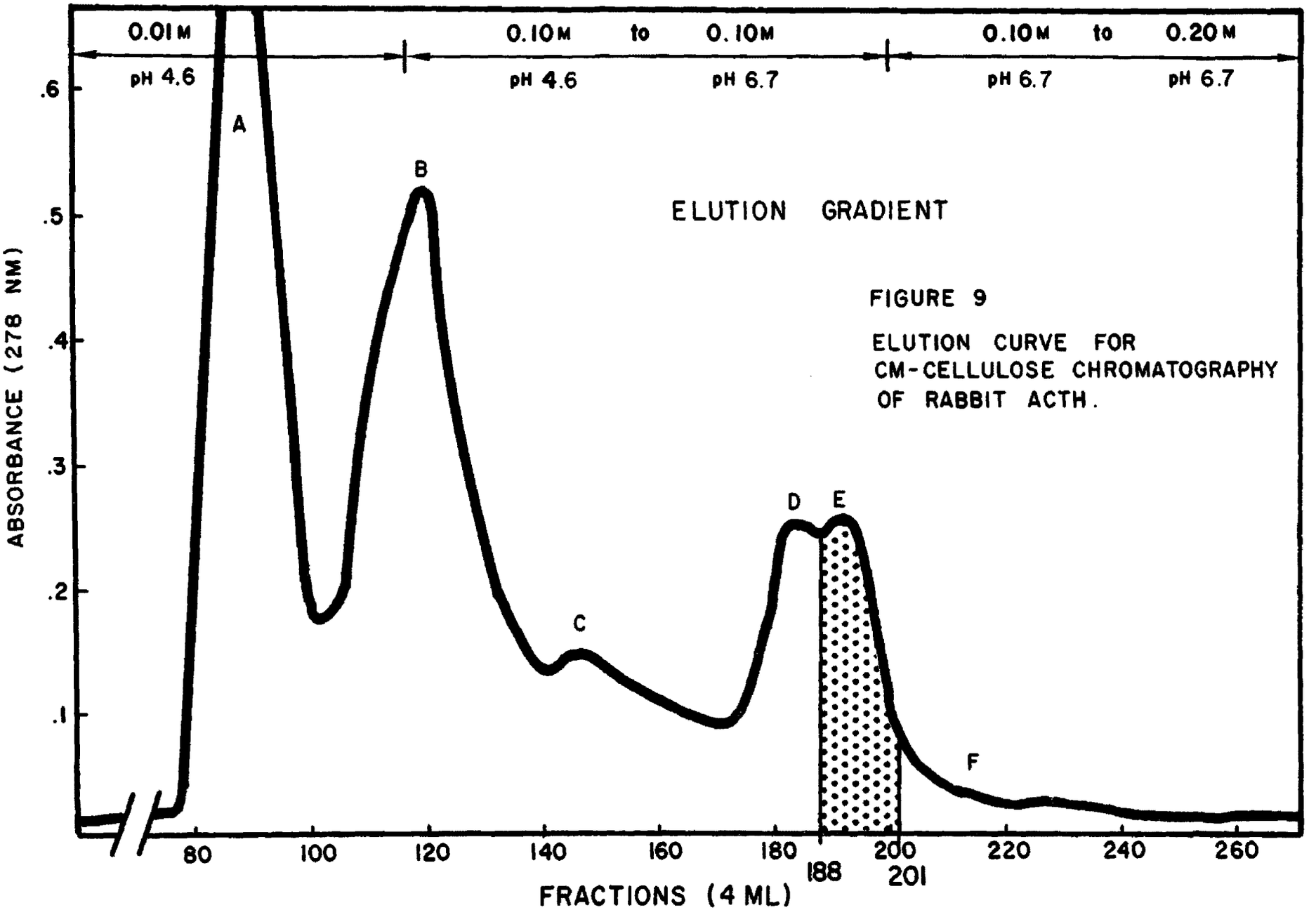

FIGURE 9

ELUTION CURVE FOR CM-CELLULOSE CHROMATOGRAPHY OF RABBIT ACTH.

ACTH Assay

The activities of the steer ACTH preparations were estimated according to the assay method of Saffrom and Schally (1965) described earlier. The standard consisted of porcine ACTH (Upjohn #7275). Both the standard ACTH and the experimental fractions were dissolved in 0.025 N acetic acid to a concentration of 9 mg/ml. A second solution was diluted 1:3 with 0.025 N acetic acid to a concentration of 3 mg/ml. Based on an activity of 62 U/mg for the standard, the standard solutions represented solutions of 55.8 U/ml and 18.6 U/ml. Aliquots of 0.1 ml used in the assay represented 5.6 U and 1.9 U or 0.9 mg and 0.3 mg of the standard and 4.5 mg and 1.5 mg of the experimental hormone. The response values ($R = \frac{O.D._{240} - O.D._{255}}{\text{Adrenal Wt.}} \times 10$) for the standard (porcine) and the experimental (steer) ACTH at both the x mg and the 3x mg level are recorded in Table 2.

The average response values at the 1.9 U and 5.6 U level for the standard procine hormone were plotted on semi-log paper. Similarly, the response values of the experimental hormone were plotted with the x mg level at 1.9 U and the 3x level at 5.6 U. Although the experimental hormone represented five times the concentration of the standard, placing the two response plots on the same cycle of the semi-log paper is permissible because $\log 5x = \log 5 + \log x$. This allows easier comparison of the two line plots. The experimental line plot is extrapolated horizontally onto the standard in order to estimate the activity of the unknown in Units. The activity and weight of the standard are known

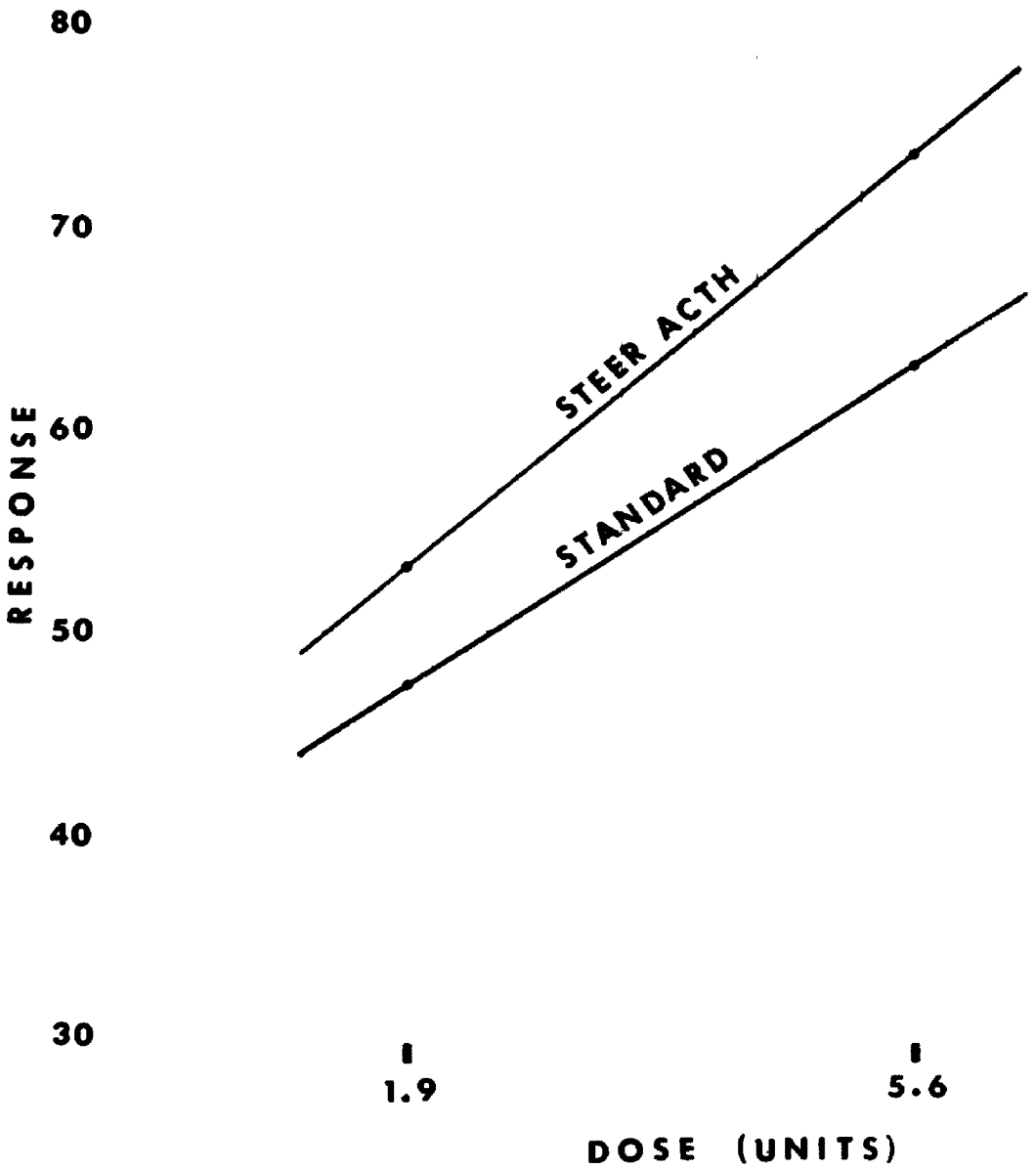

FIGURE 10 ACTH ASSAY

Response values from Table 2.

STEER ACTH: Tubes 221-260*
STANDARD: Porcine ACTH

*Steer preparation 5x concentration of standard.

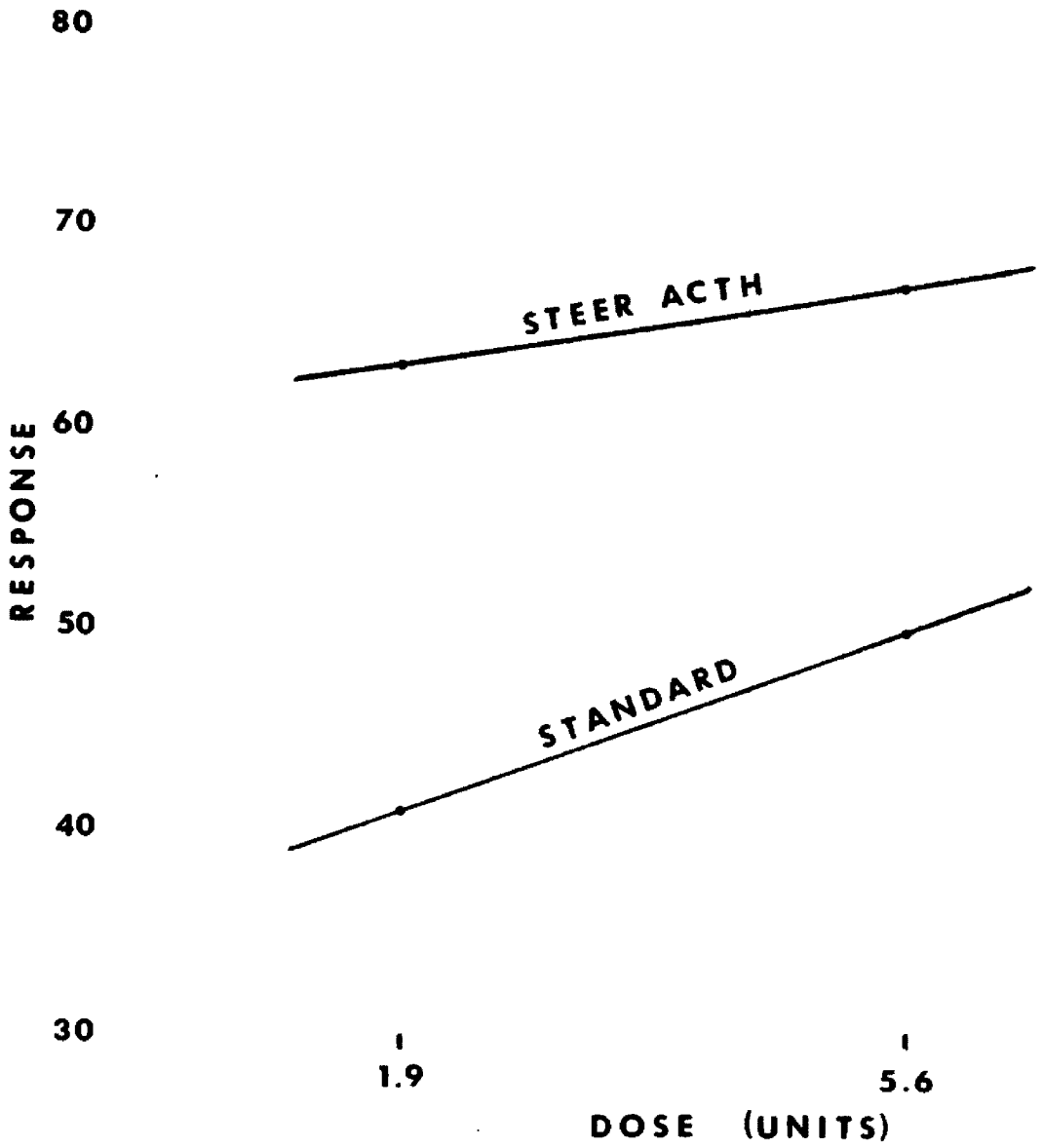

FIGURE 11 ACTH ASSAY

Response values from Table 2.

STEER ACTH: Tubes 261-304*
STANDARD: Porcine ACTH

*Steer preparation 5x concentration of standard.

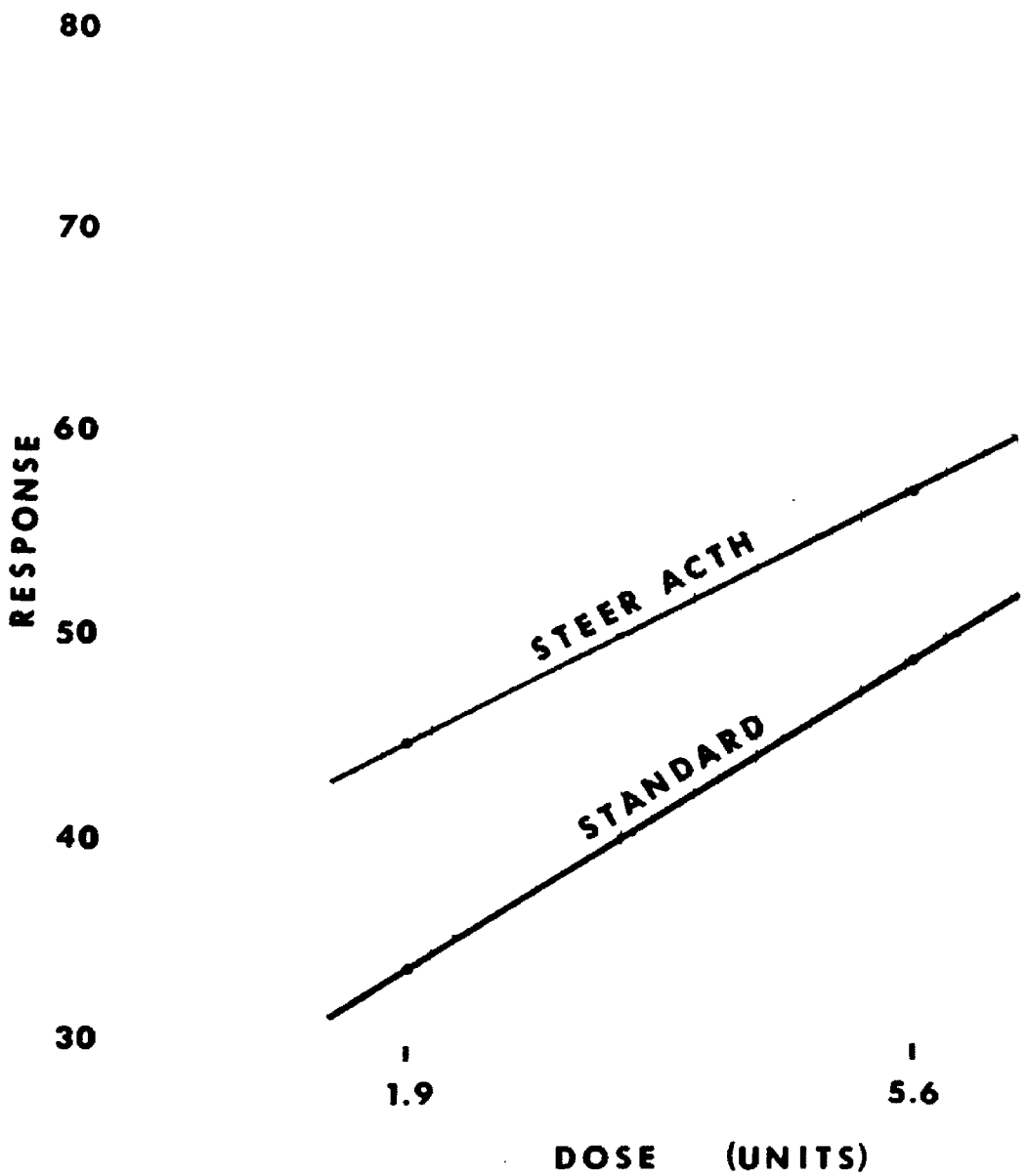

FIGURE 12 ACTH ASSAY

Response values from Table 2.

STEER ACTH: Tubes 305-350*
STANDARD: Porcine ACTH

*Steer preparation 5x concentration of standard.

TABLE 2. Response values from steer ACTH preparation assays.

CM-Cellulose Fractions		Steer 1.5mg	Steer 4.5mg	Standard 0.3mg	Standard 0.9mg
Tubes	87-120	29.4	29.0	35.9	49.5
"	121-220	31.6 16.0	44.1 21.3	34.9 23.8	47.6 25.5
"	221-260	53.1	73.8	47.1	63.0
"	261-304	63.0	66.9	40.9	49.9
"	305-350	44.3	57.3	33.2	39.9

TABLE 3. Estimated activity of steer ACTH fractions.

CM-Cellulose Fractions		Dry Weight (mg)	Approximate Activity (U/mg)	Total Activity (U)
Tubes	87-120	57.0	0	0
"	121-220	110.5	10	1105
"	221-260	19.1	15	287
"	261-304	24.4	25	610
"	305-350	10.8	25	270

and the weight of the experimental hormone is known, permitting the calculation of the activity of the experimental hormone in Units/mg. These activities are recorded in Table 3.

Similarity in slope of the semi-log plots for the experimental hormone preparation to that of the standard hormone indicates a similarity in hormone action. Taking into consideration the estimated adrenal corticosteroid stimulating activity and the similarity in the slope of the resulting response curve, the fraction made up of the eluate in tubes 261-304 was selected as the fraction most closely resembling ACTH (porcine). This fraction was estimated to have an activity of 25 U/mg, as high or higher than any other fraction, and its semi-log slope was most similar to that of the standard hormone. This fraction also corresponds to the "ACTH" peak for sheep ACTH prepared by the same method (Li and Birk, 1964). It was this fraction that was used in the rabbit adrenal stimulating experiment.

Similar assays were carried out using the rabbit ACTH fraction made up of the eluate in tubes 254-272 since it corresponded to the most active fraction in both the steer preparation mentioned above and the sheep preparation of Li and Birk (1964). However, since the results were so variable and the amount of this fraction so small (65mg), it was decided to have this preparation assayed elsewhere. With the assistance of J. W. Hinman of the Upjohn Company, it was possible to have this preparation assayed by G. W. Camiener and P. N. Tree, also of the Upjohn Company. A method of assay by C. H. Li modified by Camiener using quartered rat adrenals was used. The standard employed was Acthar (Armour). They reported an activity of 48 U/mg (Table 4) but indicated that this

TABLE 4. ACTH assay data from Upjohn Laboratories.

Sample	Average Activity Acthar Units/mg	Actual Values 4-5 dose levels in duplicate
Rabbit ACTH	48 ± 7*	55, 54, 44, 40
Porcine ACTH (Upjohn #7275)	62 ± 2	61, 63

*Average ± 1σ.

value may be lower than the true activity because of the limited solubility of the rabbit ACTH preparation.

These men also assayed the procine ACTH (Upjohn #7275) which was being used as a standard in this laboratory. The activity of this material was found to be 62 U/mg rather than 100 U/mg as previously believed. The results of their assays are reported in Table 4.

Electrophoresis

Disc gel electrophoresis on 7.5% polyacrylamide showed the porcine ACTH preparation to be more homogeneous than the other two preparations. One major band occurred at both the acid and basic pH with a second band which had a higher mobility in both systems faintly visible. The rabbit and steer ACTH preparations displayed numerous bands, but in each there was a distinct major band with approximately the same mobility as that of the porcine preparation. Figure 13 illustrates the gels run in both the acid system (pH 4.3) and the basic system (pH 9.2).

DISC ELECTROPHORESIS OF ACTH ON 7½% POLYACRYLAMIDE GEL

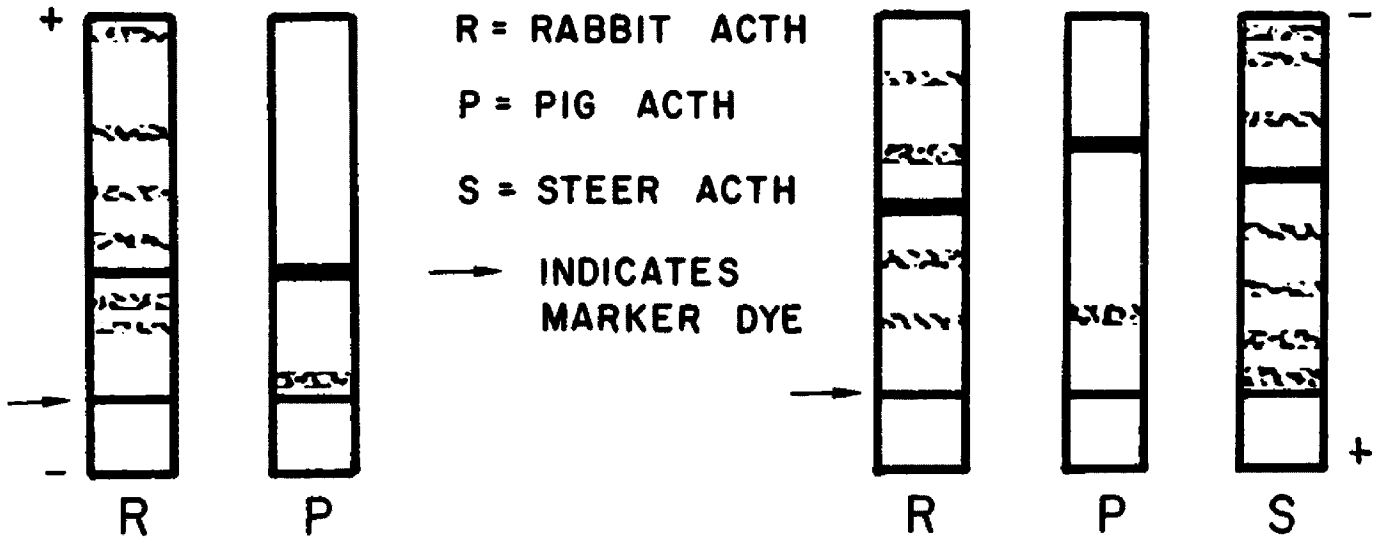

FIGURE 13

At pH 4.3 the electrophoretic mobility of the major band of the rabbit and porcine preparations were identical. No gel was run for the steer ACTH, so no comparison can be made. In the basic system (pH 9.3), the mobility of the major band of the rabbit preparation was somewhat greater than that of the procine and steer preparations. The major bands of the porcine and steer preparations varied only slightly from each other with the major band of the steer preparation having the greater mobility.

Adrenal Incubations

Tables 5 through 10 include data concerning six corticosteroids: cortisol, corticosterone, 11-deoxycorticosterone, 11-dehydrocorticosterone, cortisone, and an unidentified compound.[1] The percent recovery for cortisol, corticosterone, and 11-deoxycorticosterone based on the recovery of ^{3}H-labeled tracer amounts of these steroids added prior to extraction are reported. The mass (µg per 100 mg of adrenal tissue) for the acetylated (C-21) form of each steroid is reported. This value is corrected in all cases for dilution and has had the average value for the blank substracted from it (the blanks contained all substrate and tracer materials, but lacked the tissue). The recovered mass values and percent conversion of substrate values were also corrected for percent recovery where applicable.

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[1]This compound was believed to be 11-deoxycortisol and was treated as such. However, recent recrystallization studies by Robert Dyer of this laboratory have shown that this compound is not 11-deoxycortisol. It apparently responded to the Porter-Silber reaction for 17α-hydroxylated steroids. The experimental data is presented although the compound has not been identified and does not, therefore, contribute to the conclusions drawn in this thesis.

The individual statistics for the two major products, cortisol-21-acetate and corticosterone-21-acetate, are reported in Tables 5 and 6, respectively. Table 11 compares the average values for these two tables. The ratios of cortisol and corticosterone, both for mass and percent substrate converted, are also reported in Table 11. As expected from previous work (Fevold, 1968), these ratios in non-stimulated control tissue are very small, the average ratio for the mass being 0.01 as compared with the rabbit ACTH-stimulated tissue average of 1.2. The porcine ACTH-stimulated tissue showed a large increase in cortisol production (32.5 µg). The increase in cortisol synthesis in rabbit ACTH-stimulated tissue (3.5 µg) was not as marked as with the porcine ACTH-stimulated tissue, but, nonetheless, significantly greater than that produced in the control tissue (0.60 µg). Decrease in the synthesis of corticosterone was likewise not as great in the rabbit ACTH- as in the porcine ACTH-stimulated tissue, 30.0 µg and 26.4 µg, respectively, but it is interesting that the decrease in corticosterone production more closely approximated that found in the porcine ACTH-stimulated tissue than did the increase in cortisol production.

Tables 7 through 9 include data for three other corticosteroids: 11-deoxycorticosterone, a precursor of corticosterone, 11-dehydrocorticosterone, an oxidation product of corticosterone, and cortisone, an oxidation product of cortisol. The values for mass and percent of substrate converted in most cases is very low. 11-Dehydrocorticosterone is highest for the control tissue incubated three hours, which is also highest for corticosterone. Similarly, cortisone is highest in the porcine ACTH-stimulated tissue incubated three hours, in which the

TABLE 5. Cortisol-21-acetate biosynthesis following porcine and rabbit ACTH stimulation.

Adrenal Tissue	mg Equivalent Tissue	% Recovery[1]	µg[2] Cortisol-21-Ac	Average µg± 1s	% Conversion[3] of Substrate	Average % Conversion
Porcine	100	95.0	32.3		16.5	
3 Hour	100	76.1	37.7	32.8±4.5	16.0	16.3±0.3
	100	75.3	28.5		16.3	
Rabbit	100	37.4	4.2		3.6	
3 Hour	100	75.9	3.2	3.5±0.6	4.2	4.9±1.8
	100	62.6	3.2		6.9	
Control	100	77.0	0.8		1.5	
3 Hour	100	84.4	0.6	0.6±0.1	0.9	1.2±0.3
	100	67.1	0.5		1.2	
Porcine	100	46.7	0		0	
0 Hour	100	70.5	0.2	0	0	0
	100	77.5	0		0	
Rabbit	100	58.3	0		0	
0 Hour	100	48.5	0.3	0	0	0
	100	49.9	0		0	
Control	100	82.1	0.2		0	
0 Hour	100	72.6	0	0	0	0
	100	82.5	0		0	

[1]Adjusted such that maximum % recovery not greater than 95.5%.
[2]Corrected for dilution factor, average blank value, and % recovery.
[3]Corrected for % recovery.

TABLE 6. Corticosterone-21-acetate biosynthesis following porcine and rabbit ACTH stimulation.

Adrenal Tissue	mg Equivalent Tissue	% Recovery	µg[1] Corticosterone-21-Ac	Average µg±1s	% Conversion of Substrate[2]	Average % Substrate Conversion
Porcine	100	83.8	26.0		18.6	
3 Hour	100	90.1	25.1	26.4±1.5	17.7	18.6±0.9
	100	51.7	28.0		19.6	
Rabbit	100	89.3	27.1		23.9	
3 Hour	100	70.0	27.3	30.0±3.1	23.3	24.1±0.9
	100	63.2	35.5		25.0	
Control	100	101.5	51.7		30.3	
3 Hour	100	107.0	58.1	51.8±6.8	33.4	31.1±1.9
	100	75.5	44.5		29.7	
Porcine	100	25.0	0		0	
0 Hour	100	26.7	0	0	0	0
	100	71.9	0		0	
Rabbit	100	30.1	0		0	
0 Hour	100	45.9	0	0	0	0
	100	73.9	0		0	
Control	100	35.9	0		0	
0 Hour	100	73.2	0	0	0	0
	100	73.7	0		0	

[1]Corrected for dilution factor, average blank value, and % recovery.

[2]Corrected for % recovery.

TABLE 7. 11-Deoxycorticosterone-21-acetate biosynthesis following porcine and rabbit ACTH stimulation.

Adrenal Tissue	mg Equivalent Tissue	% Recovery	µg[1] Deoxycorticosterone-21-Ac	Average µg±ls	% Conversion of Substrate[2]	Average % Conversion µg±ls
Porcine	100	59.9	1.5		0	
3 Hour	100	24.8	4.8	3.2±1.6	0	0
	100	54.4	--[3]	(2.0)[4]	0	
Rabbit	100	63.2	1.9		0	
3 Hour	100	58.1	--	1.3±0.7	0	0
	100	54.8	0.6	(0.4)	0	
Control	100	60.9	0.9		0	
3 Hour	100	58.7	0.9	0.9±0	0	0
	100	58.1	--	(0.2)	0	
Porcine	100	68.0	1.7		4.2	
0 Hour	100	60.7	0.9	1.2±0.5	1.0	2.0±1.9
	100	67.1	0.9		0.9	
Rabbit	100	77.3	0.6		0.9	
0 Hour	100	69.3	0.7	0.9±0.2	0.9	1.0±0.1
	100	66.6	1.3		1.0	
Control	100	64.5	0.9		0.8	
0 Hour	100	72.2	0.2	0.7±0.7	0.7	1.0±0.3
	100	57.1	1.1		1.3	

[1]Corrected for dilution factor and average blank value.
[2]Corrected for % recovery.
[3](--) Indicates no value obtained.
[4]Less average zero hour value.

TABLE 8. Dehydrocorticosterone-21-acetate biosynthesis following porcine and rabbit ACTH stimulation.

Adrenal Tissue	mg Equivalent Tissue	µg[1] Dehydrocorticosterone-21-Ac	Average µg±ls	% Conversion of Substrate	Average % Conversion µg±ls
Porcine	100	119.5		12.7	
3 Hour	100	0.5	1.9±1.9[2]	13.2	12.9±0.3
	100	3.2	(1.4)[3]	12.7	(12.8)[3]
Rabbit	100	0.2		12.5	
3 Hour	100	3.8	2.4±1.9	13.5	14.1±1.4
	100	3.3	(1.5)	16.2	(14.0)
Control	100	2.4		14.3	
3 Hour	100	1.6	2.5±1.0	17.0	15.1±1.7
	100	3.6	(2.0)	13.9	(15.0)
Porcine	100	0.6		0.2	
0 Hour	100	0.7	0.5±0.1	0.1	0.1±0
	100	0.1		0.1	
Rabbit	100	1.3		0.1	
0 Hour	100	1.2	0.9±0.6	0.3	0.1±0.1
	100	0.2		0.1	
Control	100	0.4		0.1	
0 Hour	100	0.6	0.5±0.1	0.1	0.1±0
	100	0.5		0.1	

[1]Corrected for dilution factor and average blank value.
[2]Value of 119.5 µg not included in this figure.
[3]Less average zero hour value.

TABLE 9. Cortisone-21-acetate biosynthesis following porcine and rabbit ACTH stimulation.

Adrenal Tissue	mg Equivalent Tissue	µg[1] Cortisone-21-Ac	Average µg±1s	% Conversion of Substrate	Average % Conversion ±1s
Porcine	100	1.8		1.7	
3 Hour	100	1.8	1.7±0.2	1.6	1.5±0.3
	100	1.4		1.2	
Rabbit	100	0.8		0.7	
3 Hour	100	0.4	0.4±0.4	0.8	0.5±0.4
	100	0.1		0.1	
Control	100	0.4		0.2	
3 Hour	100	0.2	0.3±0.1	0.2	0.1±0.1
	100	0.2		0	
Porcine	100	0		0	
0 Hour	100	0	0	0	0
	100	0		0	
Control	100	0		0	
0 Hour	100	0	0	0	0
	100	0		0	

[1]Corrected for dilution factor and average blank value.

TABLE 10. Unidentified steroid compound following porcine and rabbit ACTH stimulation.

Adrenal Tissue	mg Equivalent Tissue	µg Unknown Compound [1]	Average µg±ls	% Conversion of Substrate	Average % Conversion ±ls
Porcine	100	2.6		0.4	
3 Hour	100	2.9	2.4±0.7	0.5	1.0±0.9
	100	1.6		2.1	
Rabbit	100	5.8		0.8	
3 Hour	100	--[2]	5.8	1.9	1.8±1.0
	100	--		2.8	
Control	100	6.2		1.2	
3 Hour	100	1.8	4.0±2.2	1.0	1.7±0.8
	100	--		2.9	
Porcine	100	0		0	
0 Hour	100	0	0	0	0
	100	0		0	
Rabbit	100	0		0	
0 Hour	100	0	0	0	0
	100	0		0	
Control	100	0		0	
0 Hour	100	0	0	0	0
	100	0		0	

[1]Corrected for dilution factor and average blank value. See text for explanation of unknown.
[2](--) Indicates no value obtained.

TABLE 11. Summary of data from Tables 5 and 6 and ratios of cortisol-21-Ac to corticosterone-21-Ac.

Adrenal Tissue	(A) Corticosterone-21-Ac Average Mass µg/100 mg Tissue	(A) Corticosterone-21-Ac Average % Conversion	(B) Cortisol-21-Ac Average Mass µg/100 mg Tissue	(B) Cortisol-21-Ac Average % Conversion	Ratio (B)/(A) Mass/Mass	Ratio (B)/(A) %/%
Porcine 3 Hour	26.4	18.6	32.5	16.3	1.2	0.9
Rabbit 3 Hour	30.0	24.0	3.5	4.9	0.1	0.2
Control 3 Hour	51.8	31.1	0.6	1.2	<0.05	<0.05

TABLE 12. Summary of data from Tables 5-9 and ratios of 17α-hydroxylated products to non-17α-hydroxylated products.

Adrenal Tissue	(A) Corticosterone-21-Ac + Deoxycorticosterone-21-Ac + Dehydrocorticosterone-21-Ac Average Mass µg/100 mg Tissue	(A) Corticosterone-21-Ac + Deoxycorticosterone-21-Ac + Dehydrocorticosterone-21-Ac Averate % Conversion	(B) Cortisol-21-Ac + Cortisone-21-Ac Average Mass µg/100 mg Tissue	(B) Cortisol-21-Ac + Cortisone-21-Ac Average % Conversion	Ratio (B)/(A) Mass/Mass	Ratio (B)/(A) %/%
Porcine 3 Hour	31.4	31.5	34.0	17.7	1.1	0.6
Rabbit 3 Hour	33.7	38.1	3.9	5.5	0.1	0.1
Control	55.2	46.2	0.9	1.3	<0.05	<0.05

cortisol production was the greatest. In both cases the rabbit ACTH-stimulated tissue is intermediate for cortisol and corticosterone, as well as their oxidation products. The reverse situation is observed for 11-deoxycorticosterone as the porcine ACTH-stimulated tissue showed the highest production of this compound, but was lowest in corticosterone.

Table 12 records the combined average values for mass and percent substrate converted to 17-deoxycorticoids and to 17α-hydroxycorticoids. As in Table 11, the ratio of 17α-hydroxycorticoids to 17-deoxycorticoids is calculated. The ratios of cortisol to corticosterone and the ratios of the combined 17α-hydroxycorticoids to 17-deoxycorticoids are very similar.

Pregnenolone-4-^{14}C not converted to other compounds is listed in Table 13 as a percent of total substrate before incubation. The sum of these values and the percent conversion of substrate values from Tables 5 through 9 gives an estimate of the percent of the substrate which can be accounted for. This value for all flasks was consistent and ranged near 50 percent.

Tables 14 and 15 include the data from the incubation of steer and porcine ACTH stimulated adrenal homogenates which was performed prior to the one described above. Since this was actually a preliminary experiment designed to test the procedures to be used with the rabbit ACTH preparation, only cortisol and corticosterone in their acetylated forms were isolated and quantitatively determined for mass and radioactivity. Tracer quantities of these two compounds were added prior to extraction in order to determine the percent of each compound recovered. Table 16

contains the average values for the mass and percent conversion of substrate to cortisol and corticosterone. It can be observed that the standard deviation of certain quantities is very great. Also, no non-stimulated controls were run since results from previous incubations would serve for comparison (Fevold, 1968). Comparison with the previously described incubation shows a definite increase in cortisol for both the porcine and steer ACTH-stimulated tissues as compared to the expected production by non-stimulated tissue.

TABLE 13. Substrate conversion and recovery after incubation and chromatographic separation.

Adrenal Tissue	Sum of % Conversion Values[1]	% Pregnenolone-4-^{14}C Recovered	Average ±1s	% Substrate Accounted for
Porcine 3 Hour	50.2	1.4 1.2 0.6	1.1±0.4	51.3
Rabbit 3 Hour	45.4	0.5 0.4 1.7	0.9±0.7	41.6
Control 3 Hour	49.2	1.0 1.2 1.4	1.2±0.2	50.4
Porcine 0 Hour	2.1	39.9 50.5 50.8	47.1±6.2	49.2
Rabbit 0 Hour	1.1	55.9 58.5 53.5	55.6±3.0	57.0
Control 0 Hour	1.0	54.5 54.5 65.8	58.3±6.5	58.7

[1]Sum of (C) and (D) from Table 1.

TABLE 14. Cortisol-21-acetate biosynthesis due to porcine and steer ACTH stimulation.

Adrenal	mg Equivalent Tissue	% Recovery	Cortisol-21-Ac[1]	Average	Conversion[2] of Substrate	Average % Substrate Conversion
Porcine	100	95.4	46.3		11.7	
#1	100	93.0	18.3	26.0±10.2	10.0	10.96
3 Hour	100	85.4	20.7	(22.7)[3]	9.3	
	100	86.4	18.7		8.7	
Porcine	100	93.1	33.1		10.5	
#2	100	100.0	16.2	19.5±6.8	12.6	10.76
3 Hour	100	87.2	11.2	(17.0)	7.8	
	100	92.3	17.6		9.4	
Steer	90	84.9	19.3	14.2±5.1	6.41	7.32
3 Hour	90	99.1	9.0	(12.9)	8.23	
Porcine #1	100	50.1	5.2	3.3±	0	0.20
0 Hour	100	90.7	1.4		0.4	
Porcine #2	100	31.8	3.9	2.5	0	0.10
0 Hour	100	86.6	1.0		0.2	
Steer	90	73.4	2.4	1.3	0.28	0.46
0 Hour	90	95.3	0.1		0.64	

[1]Corrected for dilution factor, % average blank, and % recovery.

[2]Corrected to 100% recovery.

[3]Average less zero hour value.

TABLE 15. Corticosterone-21-Acetate biosynthesis due to porcine and steer ACTH stimulation.

Source of	mg Equivalent Tissue	% Recovery[3]	Corticosterone-21-Ac[1]	Average	% Conversion[2] of Substrate	Average % Substrate Conversion
Porcine	100	57.7	0	18.9	2.1	
#1	100	57.8	1.1	(0)[3]	2.4	2.0
3 Hour	100	46.7	30.1		1.7	
	100	41.1	44.5		1.7	
Porcine	100	51.41	0		2.1	
#2	100	56.7	0	0	2.4	2.2
3 Hour	100	51.2	0	(0)	2.3	
	100	44.6	0		1.9	
Steer	90	55.4	10.9	34.1	5.3	5.4
3 Hour	90	56.5	47.3	(20.1)	5.5	
Porcine #1	100	38.5	0	22.5	0	0
0 Hour	100	31.7	45.1		0	
Porcine #2	100	41.7	23.1	55.0	0	0
0 Hour	100	19.7	88.8		0	
Steer	90	51.2	15.6	14.0	0	0
0 Hour	90	60.6	12.5		0	

[1]Corrected for dilution factor, % average blank, and % recovery.

[2]Corrected to 100% recovery.

[3]Average less zero hour value.

TABLE 16. Summary of data from Tables 14 and 15 and ratios of Cortisol-21-Ac to Corticosterone-21-Ac.

Adrenal Tissue	(A) Corticosterone-21-Ac Average Mass ug/100 mg Tissue	(A) Corticosterone-21-Ac Average % Conversion	(B) Cortisol-21-Ac Average Mass ug/100 mg Tissue	(B) Cortisol-21-Ac Average % Conversion	Ratio (B)/(A) Mass/Mass	Ratio (B)/(A) % Conversion
Porcine #1 3 Hour	18.9	20.0	26.0	9.9	1.4	5.0
Porcine #2 3 Hour	0	2.2	19.5	10.1	∞	4.6
Steer 3 Hour	34.1	5.3	14.2	7.3	0.4	1.4

CHAPTER V

DISCUSSION

To date, all studies involving corticotropin have used the ACTH molecule extracted from pituitaries of cortisol-producing species such as the pig, cow, or sheep. The work of Morozova (1966) and Fevold (1968) concerning an alteration in the ratio of cortisol to corticosterone produced by the rabbit adrenal cortex following chronic *in vivo* stimulation with ACTH was carried out using corticotropin from a cortisol-producing species, the pig. It was the goal of the present project to isolate ACTH from rabbit pituitaries and to compare the effects of chronic stimulation from this hormone on the rabbit adrenal cortex with those effects resulting from stimulation with porcine and bovine ACTH.

ACTH was first isolated from steer pituitaries in order to establish the procedure of extraction, assay, and stimulation of rabbit adrenal tissue before working with the more expensive rabbit pituitaries. Approximately 14,000 frozen rabbit pituitaries are required to provide one kilogram of tissue, whereas approximately 100 steer pituitaries are required. That a slightly greater amount of acid-acetone powder (28.6 g/kg) was obtained from the rabbit glands than from the steer (23.6 g/kg) may have been due to an inherent difference in the amount of ACTH (and other polypeptides of similar size and characteristics) present per kilogram of pituitary tissue according to species, as well as a host of other possibilities such as the condition of the animals before slaughter, the method of slaughter, the length and manner of storage of the

tissue before use, or the laboratory technique used in extraction of the ACTH fraction. Although the method of extraction was the same, some difficulty was encountered in removal of the AAP from the filter paper, especially after the suction drying in a Buchner funnel following the final acetone washing. Also, the difference in activity of these two corticotropin fractions may be attributable to one or more of the above causes. It would be of interest to determine more closely the relative amounts and activities of the ACTH in the pituitaries from these two species, but further investigations would be necessary to allow more meaningful conclusions.

In comparing the biological activity of the two preparations, Dixon (1964) suggests that even a single set of conditions does not guarantee a unique answer for the potency ratio of a pair of substances. The accuracy of slope comparison of dose-response curves for two hormone preparations is dependent upon the dose levels used. Also, the activity of samples containing the same active substance may differ even though they are similar except for minor contaminants. Dixon warns that the activity found may apply only to the assay conditions used, and to no other conditions. He further notes that since the nature of hormone action is not known, it may require several consecutive reactions as in the case of a multistep enzymatic reaction, the extent to which each of these reactions affects the whole varying with the conditions. In other words, the activities of the various ACTH fractions used in this project were determined by the in vitro rat adrenal assays and may only demonstrate the abilities of the various ACTH fractions to elicit corticosteroids under these specific conditions. The activities determined by

this means, therefore, may not reflect the relative activities of these preparations in the rabbit stimulation studies to be discussed later. Some possible reasons for variation in the relative activities of two hormone preparations under differing conditions will be considered in more detail in the discussion of structural variations of the ACTH molecule. Variations in homogeniety among the three ACTH preparations was illustrated by disc electrophoresis. The porcine ACTH was essentially pure, while the rabbit and steer ACTH preparations appeared to contain numerous proteins. Thus, on this basis it would be expected that the steer ACTH preparation, which was most heterogeneous, would be the least active and the porcine most active.

Further observations can be drawn from the electrophoretic results. Comparison of the electrophoretic mobility of the major band in each gel, assuming this band represents the ACTH polypeptide, reflects the structural similarities and variations of the molecules being compared. Although the structure of the rabbit corticotropin was not investigated, a few observations can be made from these electrophoretic results coupled with previous knowledge of other ACTH molecules from the literature. It was demonstrated that the mobilities of the major bands were similar for each preparation in both the acid and basic pH systems. Figure 1 illustrates the known primary structure of porcine and bovine ACTH molecules, as well as the structure of the human and sheep corticotropin (Hofmann, 1962). Because all corticotropins thus far studied contain 39 amino acid residues, it is likely that rabbit ACTH also contains that number. The similarity in electrophoretic mobility of the rabbit ACTH to that of steer and porcine ACTH, as well as the similarity of their elution

gradients on CM-cellulose chromatography column tend to confirm this conclusion. The primary structures of all ACTH molecules thus far investigated have been shown to vary in the region of residues 25-33 (Hofmann, 1962; Li and Oelofsen, 1967). Figure 1 illustrates that bovine ACTH differs from procine ACTH in the region of residues 27 through 31. The alanine, glutamic acid, and aspartic acid residues, positions 27, 28, and 29, respectively, of the porcine molecule can be found in positions 28, 29, and 30 of the bovine molecule. However, in positions 29 and 31 of the bovine molecule a glutamic acid and serine residue replace the glutamine and leucine residues of positions 30 and 31 of the porcine molecule. At acidic pH (4.3) the two molecules would have approximately the same net electrostatic charge and, therefore, be expected to have virtually identical electrophoretic mobility. However, at basic pH (9.3) the two molecules would differ by one net negative charge due to the γ-carboxyl group of the glutamic acid residue, position 29 of the bovine molecule, causing the bovine molecule to have a greater electrophoretic mobility. Since the major band of the rabbit preparation had the same mobility as the porcine preparation in the acid pH electrophoresis system and a greater mobility than either the steer or porcine preparation in the basic pH system, it is evident that its primary structure differs slightly from both the steer and the porcine ACTH. From these observations it cannot be determined in which region of the molecule this difference(s) occurs.

Other aspects of the ACTH molecule are also of interest. It has been demonstrated that the NH_2-terminal end of the ACTH molecule is necessary for adrenal-stimulating activity. White (1955) showed that

treatment of porcine ACTH with leucine aminopeptidase for several hours brought about an approximate 50 percent removal of the Ser and Tyr residues in positions 1 and 2, and an approximate 15 percent removal of the Ser and Met residues of positions 3 and 4 accompanied by a reduction in adrenal corticotropic activity of 64 percent. The COOH-terminal portion, on the other hand, appears much less important, since synthetic molecules consisting of 24 (Schwyzer and Kappeler, 1963), 23 (Hofmann et al., 1962), 20 (Hofmann et al., 1962), and 19 (Li et al., 1960) amino acid residues corresponding to the NH_2-terminus of the ACTH molecule exhibit high adrenal-stimulating activity. Because the ACTH molecule exists as a random coil (Li, 1962), there is no secondary or tertiary structure to be altered by variation in the length of the primary amino acid structure.

Fevold (1968) demonstrated that synthetic β^{1-24}-corticotropin had the ability to increase cortisol production in the rabbit adrenal cortex of animals stimulated for two days with chronic doses of the hormone analog. This synthetic corticotropin is fashioned after the 24 NH_2-terminal amino acid residues of porcine ACTH, indicating that all or part of the ability of ACTH to alter the biosynthetic pathway of the rabbit adrenal cortex lies in this portion of the porcine ACTH molecule. The apparent lower activity of the synthetic corticotropin for increasing the production of cortisol in Fevold's experiment may have been due to possible alterations in structure of the molecule after administration. Proteolytic enzymes might attack the smaller molecule more readily thus inactivating it more quickly, as suggested by Landon et al. (1964). Or possible variations in the rate of transport from the sight of administration to the receptor site including transport through

membrane barriers might affect the potency (Li and Oelofsen, 1967). The effect of altering the corticosteroidogenic pathway of the rabbit adrenal cortex, although significantly less than that due to native porcine ACTH, is definitely characteristic of the NH_2-terminal 24 amino acid portion of the ACTH molecule.

That the steer ACTH used in this experiment was much less pure than the porcine ACTH was observed from the electrophoretic data. The contaminants may affect the hormone molecules in some way so to account for the lower activity observed in the rabbit stimulation experiment even though the animals were stimulated with approximately the same number of units of activity according to the results of the rat adrenal assay. Therefore, the difference observed in the effect of steer ACTH on the rabbit adrenal cortex as compared to that of the porcine ACTH may be due to a difference in stability and/or solubility of the two corticotropin molecules.

The most important result of these experiments was that rabbit ACTH also showed the ability to alter the biosynthetic pathway of the rabbit adrenal cortex under conditions similar to those for the porcine, steer, and synthetic hormones. In this experiment, a greater number of units of the rabbit ACTH was administrated per animal per day than of the porcine ACTH. The resulting change in the corticosteroid production by the rabbit ACTH-stimulated tissue was less than that of the porcine ACTH-stimulated tissue. Again, this may have been due to its lesser purity or due to its lower solubility as suggested by Hinman (1969). It is evident from this that rabbit ACTH has certain active groups which resemble those of the porcine and bovine molecule, as well as of the

β^{1-24}-corticotropin. In fact, from these comparisons and previous knowledge of the ACTH molecule, it is quite likely that the NH_2-terminal 24 amino acid residues of the rabbit ACTH are the same as those of the other three molecules considered, as well as all other known structures of the ACTH molecules. However, it cannot be ruled that the 15 COOH-terminal amino acid residues play no part in this action. It is quite possible that the degree to which the ratio of cortisol to corticosterone is altered with identical dosages under identical conditions is a function of certain amino acid side-groups in this region, rather than or along with variations in solubility and/or susceptibility to degradation of the ACTH molecule mentioned above. Further, it is likely that the ACTH molecular structure differs no more between cortisol producing species and corticosterone producing species than among the various cortisol producing species. It is evident from these experiments that the differences in corticosteroid output by the adrenal cortex is due to a difference in the composition of the adrenal tissue rather than the specific structure of the corticotropin molecule.

Thus, it is seen that steer and rabbit ACTH affect the rabbit adrenal cortex after prolonged stimulation just as porcine ACTH had previously been shown to affect it. Based on his findings that Δ^5-pregnenolone is a better precursor of cortisol than is progesterone in ACTH-stimulated tissue, Fevold (1967) suggests that the shift from corticosterone to cortisol production is due to an increased 17α-hydroxylast activity, but without an accompanying decrease in 21-hydroxylase activity as suggested by Kass et al. (1954). Following the time course of the metabolism of pregnenolone-4-^{14}C by homogenates of adrenal tissue

from ACTH-stimulated rabbits, Fevold (1969) presented direct evidence for the existence of a cortisol biosynthetic pathway which bypasses progesterone with 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and 11-deoxycortisol as intermediates between pregnenolone and cortisol. These data do not support the hypothesis of Yudaev and Morozova (1965) that the increased cortisol production by ACTH-stimulated rabbit adrenal tissue was due to partial inhibition of the 21-hydroxylase, making more progesterone available for 17α-hydroxylation. A scheme of the biosynthetic pathway from Δ^5-pregnenolone to cortisol and corticosterone is presented in Figure 14.

Selye (1946) hypothesized the central homeostatic role of the pituitary-adrenocortical axis, or "general adaptation syndrome," in an attempt to explain the ability of an organism to adapt to sustained injurious or noxious environmental changes through mobilization of its adrenocortical hormones, the peripheral influences of which aid in this adaptation. Exhaustion of this adrenocortical function would terminate this ability of the organism to sustain its physiologic adaptation, and under continued stress, could lead to the death of the organism. The mechanism of the rabbit adrenal cortex discussed in this thesis may play a role in this adaptation syndrome, if it does indeed exist under natural conditions, i.e., if under extreme stress such as traumatic shock, exercise, infectious disease, hemorrhage, starvation, exposure to cold, heat, oxygen-lack, or burns, radiation, or overcrowding the rabbit pituitary releases endogenous ACTH at sufficiently high levels for sufficiently long periods of time as to bring about an increased cortisol production. This would be advantageous to the animal under

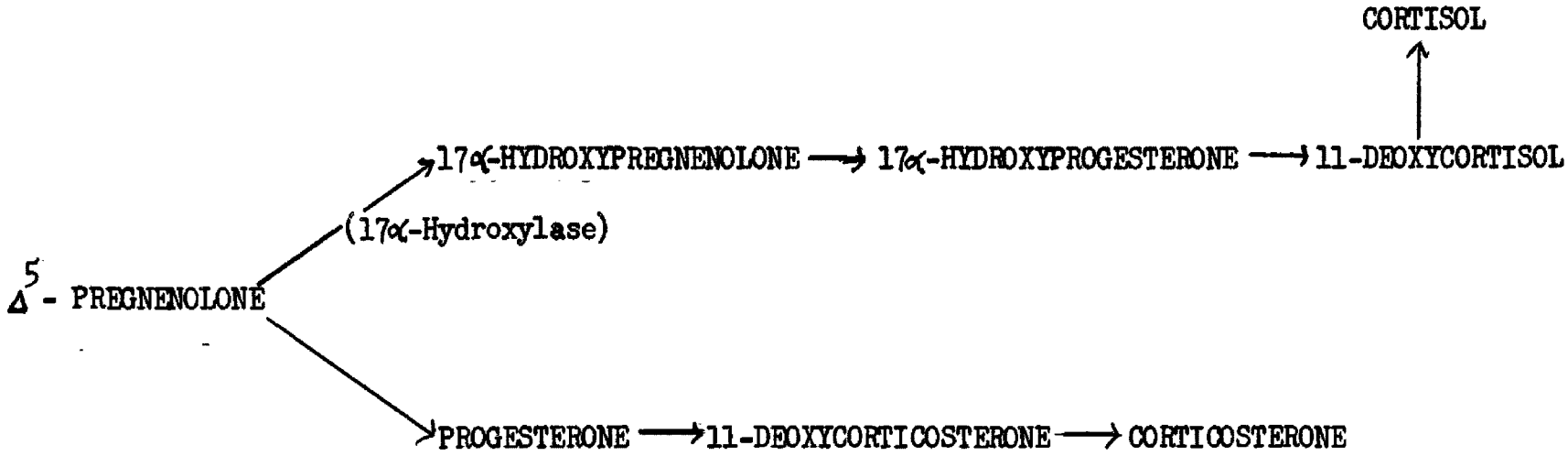

FIGURE 14. Biosynthetic pathways of the rabbit adrenal cortex.

stress because cortisol and cortisol-like compounds (e.g., cortisone) are more potent than corticosterone and 11-deoxycorticosterone in certain functions of glucocorticoids such as carbohydrate regulation, anti-inflammatory action, and immune response (Gorbman and Bern, 1962). A switch to a more potent hormone would require production of less hormonal material to meet the demand due to stress, offering protection of the adrenal tissue from exhaustion.

CHAPTER VI

SUMMARY

1. ACTH was isolated from frozen steer and rabbit pituitaries.
2. The activity of the steer and rabbit ACTH was estimated by comparing the increase in Δ^4-3-ketosteroid production by quartered rat adrenals with that produced by the porcine ACTH standard of known activity.
3. The electrophoretic mobility of steer, rabbit, and porcine ACTH were compared under basic (pH 9.2) and acidic (pH 4.3) conditions. The preparations were found to vary in number of electrophoretic bands and hence purity. The mobility of the major bands, assumed to represent the ACTH polypeptides, were similar, yet not identical.
4. Rabbits were injected intermuscularly with approximately 40 Units of rabbit ACTH or 26 Units of steer or porcine ACTH twice daily for two days. Controls received only the carrier vehicle.
5. The adrenal from similarly stimulated animals were pooled, homogenized, and incubated with appropriate cofactors in a physiological buffer at 37.5°C for two hours. Δ^5-Pregnenolone-4-^{14}C was used as substrate.
6. The resulting corticosteroids were extracted with organic solvents and separated by a series of descending paper chromatograms. Products were acetylated at position 21 prior to final chromatography.
7. The corticosteroid acetates were analyzed by the method of Porter and Silber or the Blue tetrazolium method to determine the mass of

each. Also, liquid scintillation spectrometry was performed to determine the percent of substrate conversion.

8. It was found that steer, rabbit and porcine ACTH all caused an alteration of the biosynthetic pathway of the rabbit adrenal cortex. Control tissue produced corticosterone with only trace amounts of cortisol, whereas the ACTH-stimulated tissue showed a definite increase in cortisol production with a subsequent decrease in corticosterone, indicating the activation of a 17α-hydroxylase system.

9. It was demonstrated by this that the increase in cortisol production by the rabbit adrenal cortex after prolonged stimulation with ACTH from a cortisol-producing species, i.e., the pig, was not due to the source of the ACTH, but rather a phenomena common to steer ACTH, also a cortisol producing species, and rabbit ACTH, a corticosterone producing species.

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