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January 1969

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KINETICS OF ADRENOCORTICOTROPIC HORMONE INACTIVATION IN THE DOG

ABSTRACT

Robert A. Everson, P. Dunlap Smith and Ernest L. Dobson

It is known that adrenocorticotropic hormone (ACTH) disappears very rapidly from the circulation. The rate of degradation forms an important determinant in the constancy of the circulating hormone level, and it is this aspect of ACTH inactivation in the dog to which this study has been directed. In the absence of a direct method of analysis, information of ACTH activity was limited to the effect of the hormone on the adrenal output of 11-hydroxycorticosteroids assuming a proportional relationship.

Comparable levels of circulating ACTH in beagles and mongrels were infered from plasma 11-OHCS measurements, and a circadian periodicity of 11-OHCS concentration similar to that in other diurnally active animals was observed. High hormone levels occurred at about 8:45 AM, and these declined throughout the day to low values at about midnight. Barbiturate anesthesia produced depression of ACTH release, and while the blocking action was not overcome by minor surgery, more extensive surgical maneuvers elicited near-maximal adrenocortical responses.

After hypophysectomy, adrenal 11-OHCS output declined very rapidly, indicating an ACTH half-life of 3.5 minutes and a 95% confidence limit range of 2.5-5.5 minutes. After loss of 90% of the ACTH activity, a second, slower component of degradation was apparent. This second component, with an ACTH $T_{1/2}$ of 45 minutes, was interpreted as movement of the hormone from the lymph and extravascular spaces of the "outer pool" into the circulation. A more prolonged, two-component pattern for disappearance of 11-OHCS after hypophysectomy was also observed. This produced 11-OHCS $T_{1/2}$ values of 76 minutes and 170 minutes respectively for the two components.

Graded increases in adrenal 11-OHCS output in hypophysectomized dogs were produced with graded rates of ACTH infusion between 0.05 and 0.85 mU/min. The latter evoked maximal adrenal stimulation and no further steroid elevation was produced with higher ACTH infusion rates. 11-OHCS overshoot responses were observed with sudden increases in ACTH stimulation. ACTH infusion rates between 0.01 and 0.2 mU/min induced elevations of 11-OHCS output which appeared to be proportional to something intermediate between the ACTH dose and the logarithm of the ACTH dose.

Adrenal blood flow was not significantly affected by depression of circulating ACTH after hypophysectomy.

Rapid inactivation of ACTH indicates metabolism of the hormone in blood or in an organ with a large blood flow. For instance, due to the limitation of blood flow, complete inactivation of ACTH by the adrenals could not result in a half-life of less than 70 minutes.

Blood is not the primary inactivator of ACTH, although some hormone degradation is probable. No loss of ACTH activity was noted with passage of the hormone through the liver, and the kidneys did not exhibit detectable catabolism of the pituitary peptide. Failure to demonstrate loss of corticotropic activity in systems most reasonably capable of such action leads to the speculation that metabolism of ACTH occurs in many tissues, each with a low efficiency, such that significant arterial-venous hormone differences are not detectable.

TABLE OF CONTENTS

LIST OF FIGURES

Page

Page

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone Corticotropin Corticotrophin
11-OHCS	11-hydroxycorticosteroids
17-OHCS	17-hydroxycorticosteroids
Compound B	Corticosterone
Compound F	Cortisol Hydrocortisone
kg	Kilogram
mg	Milligram
µg	Microgram
I. U. (U)	International Standard Unit International Unit
mU	Milliunit
µU	Microunit
ml	Milliliter
min	Minute
SD	Standard Deviation
SEM	Standard Error of the Mean
SEE	Standard Error of Estimation

INTRODUCTION

Pituitary effect on adrenal structure and function was recognized in 1912, when Ascoli and Leganani observed profound atrophy of the inner zones of the adrenal cortex in hypophysectomized dogs (8). Medical curiosity in the hypophyseal-adrenal relationship was further fostered by evidence of adrenal atrophy in patients with accidental pituitary damage and by recognition of the diametric effects of pituitary disorders in the production of Addison's disease and Cushing's syndrome (8). The hormonal basis of the hypophyseal influence was suggested in the classic paper of P. E. Smith in 1930, describing parapharyngeal hypophysectomy in the rat and demonstrating that daily anterior pituitary homotransplants restored to normal, or near normal, adrenal changes produced by hypophysectomy (2).

In 1942 and 1943 Li, Evans and Simpson (99, 100) and Sayers, White and Long (167) independently described the isolation of a highly purified hormone from sheep and pig pituitaries. The name adrenocorticotropic hormone (ACTH) was proposed in 1943 by Li and Evans for the active principle of the pituitary which promotes repair of adrenal atrophy in hypophysectomized animals (98), and the term corticotropin was formally adopted in 1951 for preparations possessing ACTH activity (1).

With more sophisticated methodology in protein chemistry analysis, the structures of ACTH in pig, sheep, cow and man were elucidated, a single polypeptide chain of 39 amino acids being common to all, with some species variation. The amino acid sequence 1 to 24 is identical in corticotropin of the four species. Loss of the N-terminal amino acid, serine, or cleavage of bonds through amino acid 19 results in complete loss of biological activity, but loss of amino acids 20 through 39 produces little or no effect on biological potency. Extensive reviews and discussions of the chemistry of adrenocorticotropic hormone have been presented by Li (92, 93), Evans, Sparks and Dixon (4), Hofman and Yajima (85) and Behrens and Bromer (11), and surveys of accumulated knowledge concerning corticotropin have been published recently by Sayers (165) and by Guillemin (66).

The pituitary releases ACTH in such small quantities that prior to the development of peptide concentrating techniques, it was not possible to detect corticotropic activity by direct extraction even in large volumes of plasma. Concentration estimates of ACTH in plasma are in the range of 0.1 to 1.0 mU/100 ml, with remarkable species similarities under a variety of conditions. While circadian periodicity causes fluctuations in the circulating levels of ACTH, evidence supports the concept of continuous rather than intermittent release of the hormone (41, 165, 124).

The normally low levels of ACTH release and the regulation of constancy in the limited physiological range are largely dependent on negative feed-back control from 17-hydroxycorticosteroid secretion by the adrenals (40, 208). In view of the concept of a continuous basal secretion of ACTH, an important aspect in the constancy of a low circulating level is the inactivation or degradation of the pituitary peptide. It is this aspect of ACTH inactivation to which this study has been directed.

In 1953, Syndor and Sayers found inactivation of endogenous ACTH in rats to be extremely rapid, as demonstrated by an estimated half-life of one minute (181). In contrast, Sweat and Farrell, in 1954, measured ACTH activity by fluorescence of hydrocortisone (cmpd F) and corticosterone (cmpd B) in adrenal venous blood of dogs after hypophysectomy and described a distinct drop in steroid level by 30 minutes after hypophysectomy (180). Corticosteroid output then continued to decline to about half the pre-hypophysectomy value after 1 hour, and a low, apparently constant level of secretion was attained after 3 hours. The declines of cortisol and corticosterone after hypophysectomy were essentially parallel.

While considerable effort has been exerted toward the resolution of the problem, the mechanism(s) responsible for the rapid inactivation of ACTH and the site(s) of degradation remain ill-defined (41, 57, 96).

It has been proposed that three possible modes of corticotropin inactivation may exist: 1.) utilization by the target organs, i.e., the adrenals, 2.) binding and degradation by some other particular tissue or by tissues in general, and 3.) excretion from the body in the sweat, urine or feces (116).

One of the major problems in the quantitation of circulating ACTH has been the lack of a direct analytical method for the hormone assay. While the development of immuno-assay techniques holds great promise for more precise delineation of the ACTH value spectrum, the procedures as yet are too complex and time consuming to be practical for rapid evaluation of a large number of samples (50, 109, 165, 205). A variety of indirect methods for quantitative determination of ACTH activity have been employed: depletion of adrenal ascorbic acid and cholesterol, in vivo and in vitro adrenal steroidogenesis, adrenal weight change, eosinopenia, melanophore stimulation, thymicolymphatic involution, and intracellular and epidermal lipid depletion (41, 96, 102, 165). Of these, ACTH-induced depletion of adrenal ascorbic acid and stimulation of steroidogenesis have been the most widely used, although less reliance has been placed on the former because of the uncertainty of the corticotropin-ascorbic acid interrelationship. It has been reported that depletion of adrenal ascorbic acid is not a reliable index of ACTH activity (66). In the present study, in vivo 11-OHCS secretion, as measured in adrenal venous blood, was employed as the index of ACTH activity.

ACTH has been considered to be the only stimulus of secretion of 17-hydroxycorticosteroids from the adrenal cortex of hypophysectomized animals (130). Several groups of investigators, however, have reported the direct effect of angiotensin on the secretion of aldosterone and 17-OHCS in dogs, with conflicting results in rats (5, 20, 59, 171). It has been observed that angiotensin II does not deplete adrenal ascorbic acid in hypophysectomized rats, thereby raising the question of a possible difference in hormone action on adrenals in rats and dogs (21, 65, 77). Renin has been shown to stimulate the release of aldosterone and corticosteroids from the adrenals of hypophysectomized and hypophysectomized-nephrectomized dogs (5, 20, 123).

In analyses of adrenal cortical secretions of a number of species, corticosterone predominates in the rat, mouse and rabbit, corticosterone and cortisol are about equal in the cow, ox and ferret, and cortisol is the dominant product in man, monkeys, dogs, cats and sheep (17, 18, 43, 44, 77, 79, 146, 210). While an adrenal secretion ratio of compound F to compound B as great as six has been reported in dogs (17), greater reliability has been extended to measurements of F to B ratios of 1:1 to 2:1 (43, 44, 210).

The Mattingly method employed in this study for analysis of plasma 11-hydroxycorticosteroids does not distinguish between cortisol and corticosterone (109), and thus, reference to adrenal steroid output

as an index of ACTH activity will include both of these compounds. Aldosterone is also measured by this technique although its level of secretion is quite small relative to that of cortisol and corticosterone. Chromagens measured by the Nelson and Samuels method of free corticosteroid analysis (131), using the Porter-Silber reaction as an endpoint (151), are referred to as 17-hydroxycorticosteroids (26, 131), and compound F, or cortisol, is the chief compound measured by this method (131).

Another concept to be borne in mind is that although adrenal 11-OHCS output is the reference of measurement throughout this study, the main consideration is the change in circulating ACTH levels indicated by the 11-OHCS values during the various experimental manipulations. Therefore, emphasis should not be concentrated on the kinetics of adrenal secretions unless specified, but rather a shift in thought must be maintained with reference to the kinetics of ACTH as indicated by its effect on the secretion of adrenal corticosteroids.

EXPERIMENTAL METHODS AND MATERIALS

Anesthesia

Anesthesia in all dogs, with the exception of five, was accomplished by intravenous injection of sodium pentobarbital[1] at a dosage of 30 mg per kg body weight. Periodic supplementary anesthesia was necessary in all cases in which the experiment lasted for time periods greater than about an hour.

Five dogs were anesthetized and maintained on a combination of sodium thiomylal[2] and methoxyflurane,[3] and in future experiments this would be the preferred anesthesia. Atropine was administered as preanesthetic medication to prevent salivation and exudation of fluids into the respiratory tract, although this is reportedly not a problem in the use of Metofane (11, 117). Sodium thiamylal is a rapid, ultra-short acting barbiturate anesthesia and was administered intravenously at a dose of 40 mg per lb, or 18.2 mg per kg, about 30 minutes after atropine. Individual sensitivity to Surital was found to be greater than that

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[1]Diabutal: Diamond Laboratories, Inc., Des Moines, Iowa.

[2]Surital: Parke, Davis and Company, Detroit, Michigan.

[3]Metofane: Pitman-Moore, Indianapolis, Indiana.

to Diabutal such that compensations in dosage had to be made for the proper depth of anesthesia.

With the onset of laryngeal paralysis, endotracheal intubation was performed, and the endotracheal tube was joined to an auto-inhalation apparatus whereby anesthesia could be maintained and controlled on an atmosphere of Metofane and pure oxygen.

Metofane (2, 2-dichloro-1, 1-difluoroethyl methyl ether) is a relatively new inhalation anesthesia. A brand of methoxyflurane, Metofane is a non-explosive anesthetic of low toxicity. A wide margin of safety exists between surgical anesthesia levels and toxic levels, and recovery is of a relatively short duration (11, 117).

Hypophysectomy

The operative procedure for hypophysectomy in the dog was essentially that of Markowitz and Archibald (107, 108) and of McLean (113). After induction of anesthesia the dog was placed on a dog board in a supine position. A wide oral exposure was presented with a gag device consisting of two thin metal rods supported laterally by two ring stands. The two rods were positioned behind the canine teeth of the upper and lower jaws such that appropriate adjustment of the rods along the ring stand would maintain the head in a horizontal position while opening the lower jaw for maximal palatal exposure. Heavy surgical suture was

then passed through the tip of the tongue, the tongue was withdrawn, and the suture was tied to a clamp on the upper horizontal bar. A wet gauze pad was wrapped around the tongue to prevent drying. Because of the acute nature of the experiments, no antiseptic precautions were necessary.

Palpation of the oral exposure revealed the soft palate and the palatine bone. By means of an electro-surgical cauterizing knife, the soft palate was incised lengthwise caudally from the rostral border of the palatine bone for a distance of 3 to 4 centimeters, and a retractor was introduced to expose the nasopharyngeal mucoperiosteum. Laterally the hamulate processes of the pterygoid bones were apparent, and the center of an imaginary line between the processes designates the area of the mucoperiosteum to be cleared by cauterization. Upon clearing of the mucoperiosteum the intersphenoid suture becomes visible, and this marks the avascular anterior border of the sella turcica. Even though this term is applied, it should be borne in mind that in the dog a complete sella turcica does not exist (113). The osteal incision through the outer basal plate of the skull and the cut through the diploe and inner table was accomplished by a "pear" dental burr 5 mm at the shank and 3 mm at the rounded tip. The resulting tapered osteal bore was found advantageous for better observation. Because of the close proximity of the circular sinus laterally and posterior to the sella, it was desirable

to guide the bore more rostrad. With a thin layer of the inner table still intact, it was possible to see the exact location of the hypophysis in relation to the circular sinus and to complete the drilling procedure without undue bleeding. It was necessary at times to pack the opening with bone wax to stop osteal bleeding, but the accumulation of bone dust usually enhanced clotting. When the bleeding was light, Oxycel[1] was desirable over bone wax, since the latter tended to obstruct the field if small pieces remained. The final opening of the inner osseous table was enlarged by means of a curette. A vacuum aspirator was necessary at all times to clear the field of bone debris and blood.

With only the dura mater encapsulating the hypophysis, an incision was made with a curved dural knife whereupon the hypophysis bulged forth into the osseous canal. Unlike previous reports, it was found that with suction the pituitary may not come away as a single unit, but rather in fragments, thereby leaving some question as to the completeness of the hypophysectomy. Consequently, it was found that lifting the hypophysis with suction and then cutting the stalk with the dural knife resulted in less fragmentation and more consistency in completeness of the operation than did removal of the hypophysis by suction alone. Bone wax was then tamped into the rather spacious cavity. Occasionally the

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[1]Oxycel; Oxidized cellulose, cotton type: Parke, Davis & Co., Detroit, Michigan.

venous sinus was broken during the sectioning of the stalk, and bone wax had to be quickly applied to prevent undue bleeding.

Cannulation of Blood Vessels

Cannulation of the Jugular Vein

The purpose of cannulating the jugular vein was for ready sampling of peripheral blood and for infusion of supplementary anesthesia. Following anesthesia, the dog was placed on the dog board in a supine position and pressure was applied to the jugular veins at the lateral tracheal boundaries at the cranial border of the shoulder. The distended jugulars were then palpated cephalad. The jugular vein was then punctured with an Intracath needle,[1] the polyethylene tube inserted through the needle into the vein, the needle withdrawn and the system secured with tape to the side of the neck. A saline drip bottle was connected to the cannula by way of a three-way stop-cock, and after each blood sampling or infusion, both the catheter and stop-cock were flushed with saline.

Cannulation of the Cephalic Vein, Saphenous Vein, Portal Vein, and Hepatic Artery

The purpose of cannulating the cephalic, saphenous and portal veins and the hepatic artery was to provide channels for the constant

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[1]Intracath: Bardic$_R$, C. R. Bard, Inc. Murray Hill, New Jersey.

infusion of ACTH, saline, blood, or chromic phosphate. Cannulation was accomplished by introducing an 18-gauge, thin walled needle into the distended vein and threading a length of P.E. 50 tubing through the needle and into the vein for several inches. The needle was then withdrawn, the tube taped securely in place and connected with a syringe of heparinized saline by which the tube was flushed periodically until connected to the infusion pump.

Cannulation of the Lumbo-Adrenal Vein

The lumbo-adrenal vein was cannulated for sampling of blood directly from the adrenal gland for the determination of plasma 11-OHCS output. The cannulation of the right lumbo-adrenal vein was based on the method of Hume and Nelson (88). Following anesthesia the dog was placed on the dog board in a lateral reclining position, and an incision was made in the lateral aspect of the right hypochondriac region caudal to the thirteenth rib. During surgery an electro-surgical unit was beneficial in reducing blood loss. A retroperitoneal approach to the adrenal was also used.

When the field was exposed the lumbo-adrenal vein was cleared of connective tissue and small veins, if present, were ligated. A small incision was then made in the lumbo-adrenal vein and a small plastic catheter was introduced onto the vein and tied in place. The cannula was brought to the outside through a stab wound and again tied in place

(see Figure 1). A bottle of heparinized saline solution was connected to the cannula and a slow drip was maintained when blood sampling was not taking place.

A "choker" of P. E. 10 tubing was placed around the adrenal vein between the adrenal gland and the adrenal vein-inferior vena cava juncture. Each end of the "choker" loop was threaded through a section of Intracath tubing, two sections of which had been tied together with wraps of surgical suture to provide a sliding noose. This construction of a double slide rather than single noose was found advantageous to prevent twisting of the "choker" and unplanned closure of the vein. The "choker" and noose were brought to the outside through the dorsal end of the operative wound, which was then sutured. With the noose in a relaxed position, blood flowed freely from the adrenal gland through the adrenal vein into the inferior vena cava. With the noose in a tightened position, blood was blocked from flowing through the adrenal vein and passed through the cannulated lumbo-adrenal vein into the collecting tube. Most samples were collected over a period of one minute, but in cases when blood flow was reduced, blood was collected for two or three minutes.

Collection and Storage of Peripheral and Adrenal Venous Blood

Peripheral venous blood was collected as follows: 2-3 ml of blood was drawn from the jugular vein cannula into a glass syringe previously

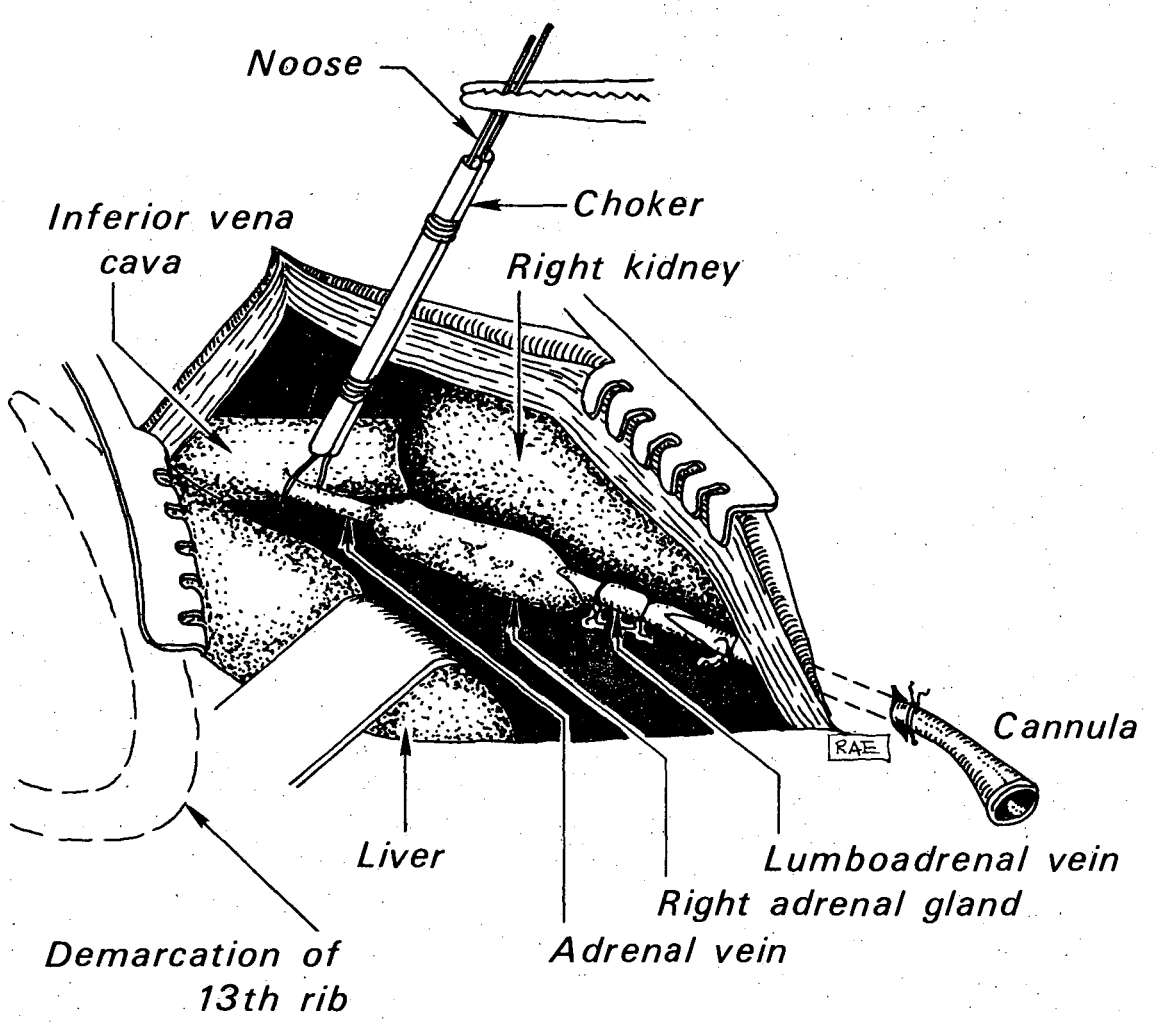

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Figure 1. Method of obtaining blood from right lumboadrenal vein. With choker in relaxed position, adrenal effluent blood flows normally through the adrenal vein into the inferior vena cava. With choker in tightened position, blood flows through the cannulated lumboadrenal vein into a chilled collecting tube. Based on method of Hume, D. M. and D. H. Nelson. Surgical Forum. 5:568, 1954.

washed with heparinized saline. A sample of 4.5-5.5 ml was then drawn into a second syringe washed with heparinized saline; this sample was immediately deposited into a graduated conical centrifuge tube maintained in a beaker of ice water, and the tube was capped. Samples in later experiments were collected in graduated polycarbonate rather than glass tubes. Blood from the first syringe was returned to the animal. In most cases blood removed from the dogs was replaced by a comparable volume from a donor animal. Hematocrits were measured periodically in Wintrobe hematocrit tubes.

Adrenal venous samples were collected as follows: the saline drip connection was removed, and the adrenal cannula was flushed with heparinized saline. After tightening and clamping the noose around the adrenal vein, blood was allowed to flow and clear the cannula for about 15 seconds. The flow for one minute, unless otherwise specified, was allowed to drop freely into a chilled heparinized centrifuge tube. At the alloted time, sampling was interrupted, the tube was capped and placed in a container of ice water, and the cannula was flushed and reconnected with the saline drip.

It was possible to collect a peripheral blood sample during the interval of collection of the adrenal vein sample.

All blood samples were maintained in ice water baths or in a frozen state at all times except during analyses or during the measurement

and transfer of plasma. Centrifugation of samples for the separation of plasma was carried out at 3-4° C.

After centrifugation, 2 ml of plasma was drawn from each peripheral sample by means of a 2 ml volumetric pipette. Total minute blood flow from adrenal samples was estimated by the graduations of the centrifuge collecting tubes, and the plasma was drawn and measured by means of graduated 1, 2, or 5 ml graduated pipettes, depending on the minute blood flow volume.

Reports are conflicting with regard to the need for refrigeration of blood samples for steroid assay, but in this study, to avoid the possibility of corticoid metabolism, plasma not analyzed on the day of sampling was frozen. It has been suggested that lack of rapid refrigeration of blood may result in steroid degradation which would make analyses as complicated as that in urine in which assay measurements are displaced in favor of the steroid metabolites (127). However, from variations in time and temperature of incubation of blood samples, it has been contended that in vitro metabolism of cortisol in whole blood is very unlikely (143). The same authors, with chromatography and radiography of added cortisol, have demonstrated that plasma stored at 25°C for 72 hours produces no steroid metabolites. Plasma refrigerated up to 2 weeks has demonstrated reproducibility of steroid levels (74, 132), and if samples are kept in the frozen state, the reproducibility of plasma

steroids has reportedly been maintained for up to 9 weeks (37, 67, 74, 94, 199). On the other hand, loss of reproducibility in corticoid analyses from frozen plasma has also been reported (13, 26).

It is known that red blood cells contain corticosteroids, but it has been estimated that 90 percent of the peripheral corticosteroids are contained in the plasma (37). Count differences between whole blood and plasma dichloromethane extracts of cortisol-$4C^{14}$ suggest that, in human blood, 75 to 80 percent of the cortisol is in the plasma fraction (143). It is possible that upon standing, the red cells lose their differential permeability, thereby allowing a greater fraction of the steroids to diffuse into the cells (37).

Replication of corticoid assay results has been obtained in both oxalated and heparinized uncentrifuged blood refrigerated overnight (198, 204). However, heparin was used as the anticoagulant in this study, and it reportedly does not interfere with steroid determinations (37, 67). In the Nelson-Samuels method for corticosteroid analysis, a hazy solution was obtained in the final reaction when citrate was used as an anticoagulant (37, 131). It is possible that some traces of calcium are carried through the solvent system and react with sulfuric acid to form calcium sulfate (37, 131).

Assay Procedure for Plasma 11-OHCS

Most methods for the determination of free corticosteroids in plasma are derived from the procedure of Nelson and Samuels (131, 169), using the Porter-Silber phenylhydrazine color reaction as an end point (151). According to paper chromatographic analysis of this method, the compound measured was demonstrated to move with what was called 17-hydroxycortisone (cortisol, hydrocortisone, Kendall's Compound F), while no evidence was found for the presence of 17-hydroxy 11-dehydrocorticosterone (cortisone, Kendall's Compound E) (132). Though this method is specific for measurement of 17-hydroxy-corticosteroids, the procedure is somewhat time consuming and requires a large volume of plasma for each analysis. In considering the number of samples required for each experiment in the present study, the use of the Porter-Silber chromagens as an indication of adrenal activity was deemed impractical.

Most fluorimetric determinations of corticosteroids have been based on the quantitative fluorescence of cortisol (11, 17, 21-trihydroxy-pregn-4-ene 3, 20-dione) and corticosterone (11, 21-dihydroxy-pregn-4-ene-3, 20-dione) in sulfuric acid as described by Sweat (179). Shortened procedures have been developed which eliminate the necessity for chromatographic separations (170) and which allow for the estimation of free corticosteroids in small volumes of rat plasma (67, 170, 195, 211).

J. van der Vies described a method for a water:carbon tetrachloride phase separation of cortisol and corticosterone (194). Since the present study is not directly concerned with the relative amounts and distinction between cortisol and corticosterone as an index of adrenal activity, it was not necessary to include a procedure to differentiate between the two.

The methods for determination of 11-OHCS in dog plasma in this study were based on techniques described by De Moor, Steeno, Raskin, and Hendrikx (26) and an abbreviated version of that method described by Mattingly (129) with recommendations by Sparks (176). All glassware used in this assay was washed thoroughly, soaked in chromic acid cleaning solution, rinsed thoroughly in tap water and then distilled water, and then dried in a drying oven.

Before the assay method of Mattingly was adopted, plasma samples from 10 animals were analyzed by the method of De Moor et al. (26). The plasma from each blood sample was diluted to 3.5 ml with distilled water and combined with 12 ml distilled hexane in a glass-stoppered centrifuge tube, and the combination was shaken vigorously for 30 seconds. Following aspiration of the hexane layer, 3 ml of the original was transferred to another tube and diluted to 5 ml with distilled water. 15 ml of dichloromethane was added, and the tube was stoppered and inverted 30 times.

Upon separation of the aqueous and dichloromethane extraction layers, the aqueous layer was aspirated and discarded, and 12 ml of the extraction layer was transferred to another tube. One ml of 0.1 N NaOH was added to each extracted sample, and the combination was shaken for 15 seconds. The aqueous layer was again aspirated and discarded, and 10 ml of the extraction solution was transferred to a clean centrifuge tube.

Concentrated sulfuric acid and ethyl alcohol in proportions of 3:1 by volume were mixed fresh in an ice bath for each batch of samples to be analyzed. The H_2SO_4:ethyl alcohol mixture was then allowed to equilibrate to room temperature. At two minute intervals, 2 ml of the acid mixture was added to each of the dichloromethane extracts, and these were shaken vigorously for 15 seconds. Caution was maintained to avoid spattering when the glass stopper was released following shaking. The dichloromethane layer was then aspirated and discarded.

Five minutes after mixing of the dichloromethane extract with sulfuric acid, fluorescence of the acid solution was read by means of an Aminco-Bowman Spectrophotofluorometer with an Aminco Photomultiplier Microphotometer attachment.[1] A straight-sided 5 ml quartz

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[1] American Instrument Company, 8030 Georgia Ave., Silver Springs, Maryland. The use of this instrument was generously provided by Dr. Norman K. Freeman of Donner Laboratory.

cuvette was used. With the exception of one change due to breakage, the same cuvette was used for the reading of all samples, thus eliminating the necessity of selecting matching cuvettes for consistency in readings. The cuvette was always oriented the same in the spectrophotofluorometer as designated by an etched mark on one of the sides. Following each reading the sample was discarded, and the cuvette was rinsed three times with distilled water, twice with acetone, and then dried over a suction tube.

The activation and emission wavelengths for 11-OHCS determination were 475 and 512 millimicrons respectively.

With the exception of the 10 animals described above, all other plasma samples were assayed for 11-OHCS according to the technique of Mattingly (109) with recommendations by Sparks (176). Two milliliter plasma samples were used for the assay of steroids in peripheral blood, whereas plasma collected over the period of one minute, and occasionally two or three minutes when adrenal blood flow was slow, was used in the assay of adrenal vein 11-OHCS. This volume was usually less than 2 ml and, in order to maintain a standard extraction volume throughout, was diluted to 2 ml with distilled water when necessary.

Fifteen milliliters of dichloromethane was added to each plasma sample in glass-stoppered centrifuge tubes. The tubes were then stoppered tightly and shaken for 10 minutes on an automatic horizontal

shaking apparatus. The upper aqueous layer was aspirated and discarded, and 10 ml of the dichloromethane extract from each tube was transferred to a clean tube.

It was recommended by Sparks that redistilled dichloromethane be used in the extraction procedure (176). Reagent grade dichloromethane, spectrograde dichloromethane, redistilled reagent grade dichloromethane and standard grade dichloromethane were run through a silica gel column and compared by infrared spectrometry. The four groups of dichloromethane[1] exhibited only slight variations in the infrared spectrograms and showed the presence of no alcohols which would affect the extraction of 11-OHCS. In addition, spectrophotofluorometry of sulfuric acid:ethanol following shaking with each of the four groups of dichloromethane showed no detectable differences. It was concluded that reagent grade dichloromethane from the Matheson Company was adequate for extraction of 11-OHCS by the Mattingly technique without further redistillation.

Some difference in background fluorescence was noted, and it seemed to be related to the batch of sulfuric acid used and the length of time after which the container had been opened. No qualitative investigation was attempted in this instance. The background fluorescence for each group of samples was consistent for that group as indicated by

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[1]The Matheson Company, Inc., Norwood, Ohio and East Rutherford, New Jersey.

fluorescence of the reagent blank and was subtracted from the fluorescence value of each sample.

The ethanol[1] recommended by Sparks was the same as had been used in all previous experiments.

Sulfuric acid and ethanol in proportions of 3:1 respectively were mixed fresh for each batch of samples no longer than one hour prior to mixing with the first sample. Both the sulfuric acid and ethanol were chilled before being mixed in an ice bath. The mixture was then allowed to cool to room temperature.

At 2 minute intervals, 5 ml of sulfuric acid:ethanol mixture was added to each of the dichloromethane extracts. The tubes were shaken vigorously for 20 seconds, and, after allowing for separation, the upper dichloromethane layer was aspirated and discarded. Fifteen minutes after mixing, fluorescence was recorded using the 5 ml quartz cuvette and the Aminco-Bowman Spectrophotofluorometer.

For determination of plasma 11-OHCS values, two sets of three cortisol standards each were exposed to the same analysis procedure as the plasma samples: a low set of 0.75 μg, 0.50 μg, and 0.25 μg as standards for peripheral plasma samples and adrenal samples after hypophysectomy and during low levels of ACTH infusion, and a higher

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[1]Rossville Gold Seal, 200 Proof, Commercial Solvents Corp., Terre Haute, Indiana.

set of standards of 10 μg, 6 μg, and 3 μg for samples before hypophysectomy and during higher levels of ACTH infusion.

The spectrophotofluorometer was supplied with a photomultiplier microphotometer unit, which consists essentially of a sensitive photomultiplier tube and an amplifier which responds to a small current produced by the photomultiplier tube and registers the amplified current on a meter. Positions of the multiplier selection switch in steps of 1.0, 0.3, 0.1, 0.03, 0.01, 0.003, and 0.001 insert precision resistors into the input circuit of the amplifier to change the sensitivity in fixed steps over a range from 1000 to 1 (2). The relative intensity of the light incident on the photomultiplier is then the product of the meter reading, the percent transmission, and the above multiplication factors.

Comparison of fluorescence readings of cortisol standards demonstrated some inconsistency of lower steroid levels recorded at different meter multiplier settings. From a total of 114 samples, factors of multiplication for equating fluorescence readings at a meter multiplier of 0.1 with readings at 0.03 for increasing cortisol levels were: 0.25 μg, 3.2 (range 2.6-3.7); 0.5 μg, 3.3 (range 3.1-3.4); 0.75 μg, 3.3 (range 3.2-3.4); and 1.0 μg, 3.3 (range 3.2-3.4).

Since fluorescence from plasma samples covering a wide range of 11-OHCS content could not be read at one given meter multiplier setting and due to inconsistent fluorescence readings at different multiplier

settings, it was decided to read all samples of higher 11-OHCS values at one multiplier setting and the fluorescence of lower 11-OHCS content at another, with standards recorded throughout the ranges of the two multiplier settings. The higher standards and pre-hypophysectomy samples were read first with the meter multiplier set at 0.1 and the activation and emission wavelengths adjusted to maximal fluorescence readings of 475 and 512 respectively. The lower standards and peripheral plasma samples were read at a meter multiplier setting of 0.01 and the same activation, emission, and sensitivity as for the higher cortisol standards and plasma samples.

A reagent blank of water was run through the same procedure as the standards and plasma samples and was read at meter multiplier settings of both 0.1 and 0.01. These readings were then subtracted from the appropriate standards and plasma values as background fluorescence.

Standard graphs were constructed by plotting the cortisol values for each set of standards versus the fluorescence reading of each minus the background reading of the reagent blank. The graphs were constructed in terms of micrograms cortisol versus fluorescence readings, and slopes were calculated from the proportional relationships. By means of the slopes of the standard curves, the fluorescence of the plasma samples, the volume of each sample, the collection time of the

adrenal samples and the weight of the animals, 11-OHCS values were calculated in terms of concentration (micrograms per milliliter plasma) or in terms of adrenal 11-OHCS output (micrograms per minute per 10 kilograms body weight). In order to establish a consistent style of presentation of data throughout the study, all graphs illustrating changes in circulating 11-OHCS and ACTH, which were frequently exponential, were constructed on a semi-logarithmic scale.

When 0.5, 1.0, 2.5 or 4.0 μg of cortisol was added to plasma, the mean recovery of corticoid using the Mattingly assay method was 97.5% with respective values of 100, 103.2, 94.8 and 91%. After addition of tritium labeled cortisol to two plasma samples assayed by the Mattingly method, activity readings indicated cortisol recoveries of 98.9 and 97.9%.[1] These values are comparable to a mean cortisol recovery of 98.2%, range 93 to 104%, recorded by Mattingly after addition of 0.5 or 1.0 μg of cortisol to plasma (109). Recovery of 4-C^{14} cortisol, 95.4 and 98.1%, did not differ significantly from the recovery from distilled water, 98.3% (109). In addition, De Moor et al. reported a mean recovery of 101.4%, range 96.5 to 107.3%, after addition of 0.125, 0.25 or 0.5 μg cortisol to plasma (26).

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[1] These data were generously provided by Miss Barbara A. Bushnell from analyses conducted at Donner Laboratory.

ACTH and Infusion Procedures

Corticotropin for constant infusion was obtained from two sources: Acthar[1] and purified porcine ACTH without added gelatin[2] (95, 137). Both lyophylized powders are readily soluble in water or saline solution, and in all studies described, saline was used as the solvent medium.

The possibility of prolongation or reduction of activity with binding of the ACTH molecule to gelatin has been reported (156). However, general observations in this study, although not quantitative, showed no difference in the onset of activity with the two types of corticotropin, and communication with the Armour Pharmaceutical Company failed to provide information on this question (49). All Acthar solutions were adjusted to a pH of 2.3-2.8 to prevent interaction of the hormone with glass, while ACTH obtained from the Hormone Research Laboratory was infused without adjustment of pH, as recommended by Papkoff (137).

Two pumps were used for the infusion of ACTH and both operated at an infusion rate of 0.17 ml/min. The pump for saline and ACTH-saline infusions consisted of a screw-driven, vertical, double syringe arrangement, such that a rapid switch could be made from the infusion of one solution to that of another.

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[1]Acthar, Corticotropin Injection: Armour Pharmaceutical Company, Box 511, Kankakee, Illinois, 60901.

[2]This ACTH was generously supplied by Dr. Harold Papkoff, Hormone Research Laboratory, University of California Medical Center, San Francisco, California, 94122.

ACTH-blood solutions were infused with a single horizontal syringe pump, equipped with a rotating magnetic mixing bar, which was found necessary to prevent settling of red blood cells. Prior to the infusions, ACTH was dissolved in a few milliliters of saline and at the appropriate time was diluted with blood from a donor dog. In switching from an ACTH-blood solution to an ACTH-saline or saline solution, it was necessary to exchange the entire syringe before resuming the infusion. Although somewhat awkward with resultant brief interruptions in the infusion, it was considered more consistent to continue the ACTH-saline or saline infusions with the same pump as that with the ACTH-blood infusions so as to forego discrepancies which might be presented in the use of a different pump.

Estimation of Liver Blood Flow:

Chromic Phosphate Infusion

The purpose for the infusion of labeled colloidal chromic phosphate, $CrP^{32}O_4$, was to detect any changes in liver blood flow during the course of the experiments, particularly as affected by hypophysectomy and ACTH infusion.

The use of colloidal chromic phosphate has been described as a tool in the measurement of liver circulation (28), and colloidal disappearance has been used extensively in the measurement of liver blood flow (30).

A single injection of colloidal chromic phosphate disappears very rapidly from the circulation according to a curve that is essentially exponential (31), and, therefore, the amount of labeled material removed at any time is proportional to the amount present at that time. This is expressed in the well known exponential equation,

$$C_t = C_o e^{-kt}$$

where C_t is the concentration at time t, C_o is the concentration at t = 0, and k is the colloid disappearance rate constant which is related to the half-time of peripheral disappearance by the equation,

$$k = \frac{0.693}{T_{1/2}}$$

A mean half-time of 1.4 min, standard deviation of 0.3 min, for the initial disappearance of chromic phosphate in mongrel dogs has been determined (30). The average $T_{1/2}$ for beagles was 1.19 ± 0.23 min, which was significantly different from that of mongrels (30).

During constant infusion of chromic phosphate, an equilibrium concentration in the blood is attained when the rate of infusion becomes equal to the rate of removal, which is proportional to the amount present. This is expressed by the equation,

$$C = \frac{I}{kV}$$

where I is the rate of infusion, V the volume of distribution, and kV the blood flow through the organ of clearance (29). When the efficiency of clearance is high as it is in the liver, kV is proportional to the blood flow, and any change in liver blood flow establishes a new equilibrium concentration of chromic phosphate.

This equation assumes 100% clearance if kV is the blood flow, and while the efficiency of $CrP^{32}O_4$ in the dog is 95-97%, for practical purposes, it is considered to be completely removed in a single passage of blood through the liver (30, 31). Thus, an increase in liver blood flow would be reflected as a decrease in peripheral blood activity, while a decrease in flow would be evidenced by an increase in activity.

Chromic phosphate for these studies was prepared according to the method described by Dobson (30, 31).[1] Infusion of chromic phosphate was carried out at an activity level of 0.5 μc/ml saline at an infusion rate of 0.17 ml/min. The infusion pump was specially designed for constancy of mixing and infusion.

At predetermined time intervals, 1 ml peripheral blood samples were drawn from the jugular vein cannula. These samples were then plated, dried, and the activity was counted.[2]

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[1]Some colloidal chromic phosphate was prepared by Miss Barbara A. Bushnell in Dr. E. L. Dobson's lab at Donner Laboratory and other preparations were obtained from Dr. Jefferson Davis, Radiocarbon Laboratory, 1012 Washington St., San Carlos, California.

[2]Sharp Widebeta Counter, Beckman Instrument Company, Richmond, California.

RESULTS AND DISCUSSION

Normal Peripheral 11-OHCS Concentration

11-OHCS concentration was determined in peripheral plasma from 29 beagles within a 3 minute period immediately prior to administration of anesthesia. All samples were obtained between 9:00 and 11:00 AM. The mean concentration was 0.26 μg/ml, range 0.09-0.38, SD 0.07. In addition, plasma 11-OHCS concentration was determined in 6 dogs, mid-collection time at 8:42 AM, as part of the demonstration of circadian periodicity, and these gave an average of 0.31 μg/ml, range 0.23-0.38, SD 0.05. The combined average of these values for the 35 animals was 0.26 μg/ml, SD 0.07, and the 24 hour average for the 6 diurnal study dogs, without regard to periodicity, was 0.27 μg/ml, range 0.18-0.38, SD 0.05.

These corticoid values are somewhat higher than the mean 17-OHCS of 11.0 μg/100 ml, SD 3.2, determined by Zukowski and Ney in 5 mongrel dogs using the Porter-Silber assay method (141, 213). Eik-Nes and Samuels, using the assay of Nelson and Samuels, found a mean of 4.0 μg/100 ml, range 2-7, in 11 mongrels previously accustomed to venesection (38, 131).

In an effort to determine if the discrepancy in values may have been attributed to a difference between beagles and mongrels, peripheral plasma 11-OHCS concentration was determined in 7 mongrels at 9:15-9:45 AM. The mean concentration was 0.24 μg/ml, range 0.17-0.28, SD 0.03, as compared with 0.26 μg/ml, determined in the beagles above, thereby showing no strain differences.

The fact that the Mattingly method of corticoid analysis measured both cortisol and corticosterone may have been a contributing factor to the higher plasma corticosteroid values recorded in this study. In addition, it has been reported that lower corticoid values have been obtained in unanesthetized dogs trained to frequent venipuncture (38), although no comparative data were presented relating this with untrained, unanesthetized animals. In view of the evidence presented in the next section concerning the elevation of plasma corticoids with various forms of trauma, differences in steroid levels between trained and untrained animals appear to be a reasonable possibility. Elevated corticosterone levels in rats have also been attributed to routine physical handling (67).

The concentration of peripheral plasma corticoids in humans has been recorded by a number of investigators with a range of values as follows: 4-10 μg/ml (132), 10.2 μg/ml, SD 1.7 (121), 10.6 μg/100 ml, SD 2.6 (16), 14.7 μg/100 ml, SD 3.95 (202), 12-15 μg/100 ml (12) and 17.4 μg/100 ml, SD 5.17 (125). Wide individual variations of corticosteroids from day to day and from week to week, as well as extreme

differences from one subject to another, have been emphasized in both dogs and man (12, 17, 78). Citing values for corticosteroid production in the dog from 120 to 3600 μg/gland/hr from several investigators, Hechter et al. noted the same range of variation in 17 dogs in their own study (78). Since exogenous ACTH failed to elevate plasma corticoid levels, the authors concluded that the adrenals were being maximally stimulated by endogenous ACTH under the conditions of study. It was postulated that the wide range of corticosteroid secretion was a reflection of the variable steroidogenic capacities of the adrenals rather than an indication of the rate of secretion of endogenous ACTH (78).

The existence of circadian periodicity must also be taken into account when considering the variability of circulating corticosteroid levels. Peripheral plasma 11-OHCS was determined in 6 beagles, 3 male and 3 female, at 4 hour intervals over a 20 hour period. The variability of the 11-OHCS concentrations in the individual animals was such as to obscure any cyclic trend; however, the averages of the respective time groups describe a cyclical pattern as shown in Figure 2.

The time for blood sampling for all 6 animals in each collection period was between 19 and 30 minutes, and the time indicated for each average concentration was the midpoint for each sampling interval.

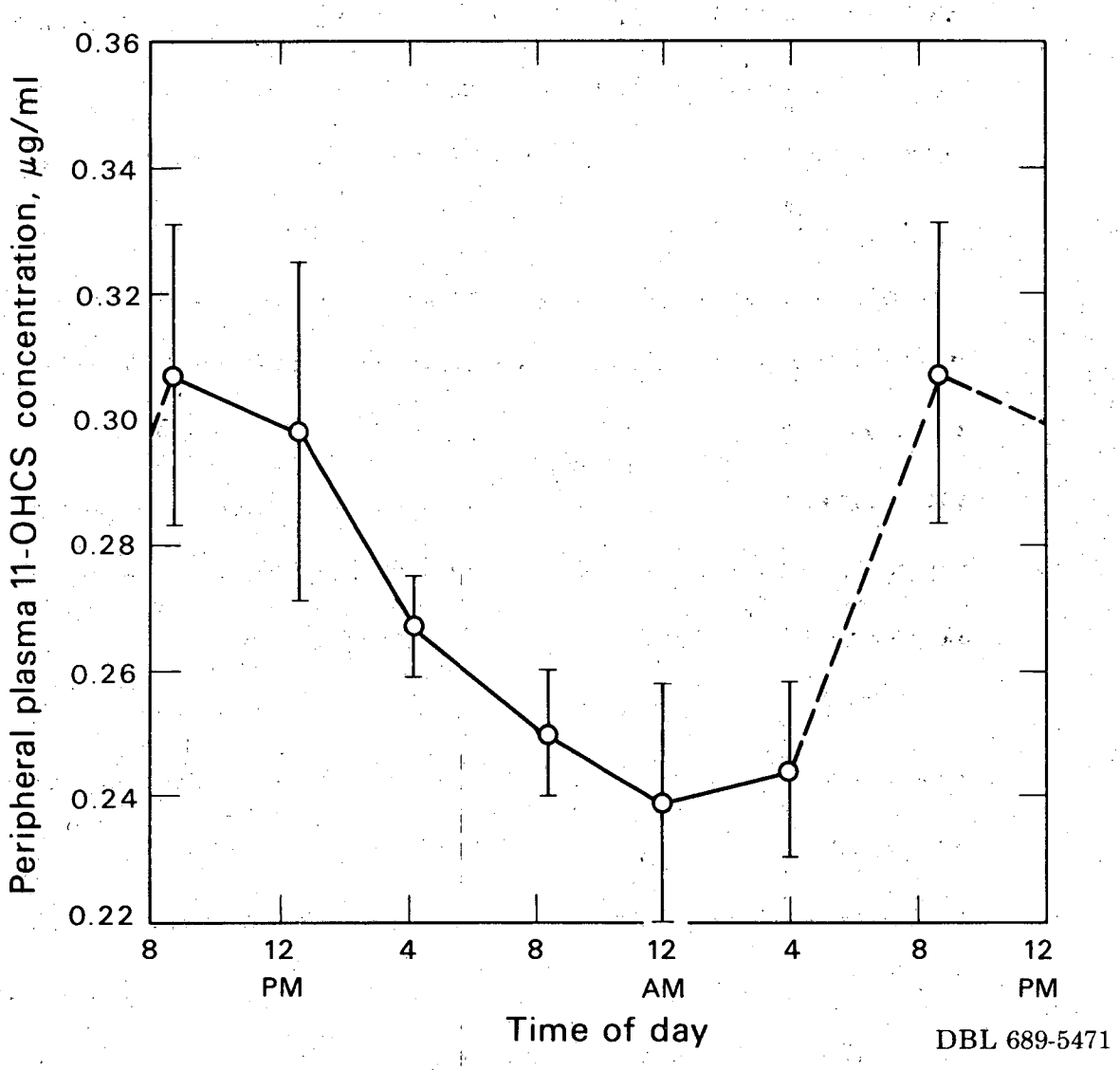

Figure 2. Circadian periodicity of peripheral plasma 11-OHCS concentration. Mean values of successive 4 minute periods (6 dogs). Vertical bars denote standard errors of the mean.

The highest 11-OHCS level as indicated by these averages occurred at 8:42 AM, with a mean concentration of 0.31 μg/ml, SEM 0.02. The level then fell progressively throughout the day with the lowest mean value of 0.24 μg/ml, SEM 0.02, occurring at 12:05 AM.

During the collection periods, particularly at night during hours of normal canine inactivity, there was some concern that movement of the animals and disturbance by other animals while removing the dogs from the holding room might interfere with the cyclic steroid picture. It is apparent that such was not the case and that periodic disturbances - waking, movement, disturbance by other animals and venipuncture - were not sufficiently traumatic to elevate 11-OHCS levels so as to obscure the circadian periodicity. That is not to say, however, that the cyclic variation may not be more pronounced under conditions of greater isolation and training of the animals.

Eik-Nes and Samuels observed in groups of dogs subjected to various forms of stress that only trained, unanesthetized animals failed to show a rise in 17-OHCS secretion throughout the 6 hour period following stress (38). The corticosteroid level in these trained animals fell from 4 μg/100 ml, range 2-7, at 8:00-9:00 AM to 2 μg/100 ml, range 1-3, at 1:00-2:00 PM. The authors suggested that this decline might be attributed to a circadian cycle, and while the steroid depression in these animals appeared to occur more rapidly than those

described in the present study, both showed a decline after the high early morning readings.

Halberg et al. noted that the circulating eosinophil level, an index of corticosteroid activity, in dogs at 9:30 AM was only about three-fourths of that at 6:30 PM (69). Variability of the levels in individual animals was not sufficient to reveal a cyclic eosinopenia, but group data demonstrated the existence of a diurnal variation.

Depression of eosinophil level occurs concomitantly with elevation of circulating corticosteroids, and the presence of a functional adrenal cortex appears to be necessary for the manifestation of a cyclic eosinopenia (70). In both mice and men, authenticated adrenal insufficiencies and the failure of the usual eosinophil rhythm occurred together (70).

The existence of a circadian rhythm in both urinary and plasma corticoid levels in man is well documented (12, 27, 133, 144, 161, 198, 199). Peak plasma steroid levels of 13-27 μg/100 ml appear to occur between 6:00 and 10:00 AM, and these fell throughout the day to a low of 3-15 μg/100 ml between 6:00 PM and 2:00 AM. The wide range for both high and low corticosteroid values may be attributed to the variability in times at which sampling occurred.

It has been observed that the standard deviation increased proportionally with mean 17-OHCS values in circadian studies in man (39);

that is, in the early morning hours, when the plasma corticoid secretion is high, the standard deviation of the average of those values is high; and in the late afternoon, when the mean corticoid values are low, the respective standard deviations are low. While no such direct relationship was noted in the present study on beagles, the wider range of standard deviations and standard errors of the mean did occur in the morning hours and the narrower range in the evening, with the exception of that at midnight.

The daily rhythm of plasma 17-OHCS in rhesus monkeys showed a pattern similar to that in man (118). The evening low, however, occurred somewhat earlier than in man, at about 9:00 PM, with a high at about 6:00 AM. The level fell sharply until about 9:00 AM and then declined slowly throughout the remainder of the day. Although no samples were obtained between 4:00 and 8:00 AM, dog data in the present study indicate that the 11-OHCS level declined rather slowly from the 8:00 AM high until about noon, when a sharper drop ensued. This fall continued until about 4:00 PM with a somewhat less steep reduction until 8:00 PM. The group mean of the 11-OHCS concentrations indicates a low at about midnight.

The 17-OHCS values in monkeys, averaging 37 μg/100 ml plasma, are reportedly considerably higher than those in the dog, mouse, rat and man (118), and the 24-hour corticoid urinary values in monkeys ranged

from 21.0 to 56.3 μg/100 ml. While not as high as that reported in monkeys, the high corticoid values recorded for beagles in this study, 0.31 μg/ml, range 0.23-0.38, were higher than those reported above for dogs and man. The 24-hour mean low, 0.24 μg/ml, range 0.18-0.32, while comparable to that in monkeys, was still somewhat higher than values reported for man and dogs. The eosinophil count of monkeys has also been found to correspond with the circadian variation of both dogs and man, with a low at about noon and a high at 9:00 PM (118).

The cyclic variation of corticosterone and eosinophil levels in rats and mice, nocturnally active animals, is the reverse of that seen in the dog, monkey and man (118, 171, 185). The highest corticosteroid levels and the lowest eosinophil counts in the former animals occurred at 4:00 PM, with a reversal in values at 4:00 AM. It was concluded that the magnitude of variation was such as to have a significant bearing on the measurement of "resting" corticosteroid levels (118).

In man, 70% of the daily basal corticosteroid secretion occurred between midnight and 9:00 AM (27). Thus, the major portion of the adrenal secretion was during sleep and during the early morning hours in an apparent "anticipation" of day-time activity. In the circadian rhythm of 11-OHCS in beagles, the steepest rise of 11-OHCS concentration, estimated from the initial 8:00 AM readings, occurred between 4:00 and

8:00 AM. From integration of the circadian 11-OHCS curve, it was approximated that 66% of the daily 11-OHCS secretion occurred between 5:00 AM and 2:00 PM.

The cyclic changes in nocturnally active animals, the reverse of that in the dog, monkey and man, are characterized by the same rise in corticosteroid levels prior to periods of activity. Whether this period of "anticipation" represents an accumulation of steroids in the hours preceding activity (which is possible since the half-life of adrenal corticoids is relatively slow), an increased production during those hours, or an increased production and decreased metabolism of corticoids, has not been elucidated. It should be noted in passing, however, that variation of adrenal steroid secretion is not limited to the more commonly recognized diurnal cycles, but has also been observed as an annular periodicity. Macchi and Hechter have demonstrated in perfused bovine adrenals maximally stimulated with ACTH that the mean corticosteroid secretion in the winter, 352 $\pm$ 21 μg/gland/hr, was significantly greater than the yearly low of 121 $\pm$ 30 μg/gland/hr in the summer (103).

Ney et al. have established in man, at least, that the daily cyclic variation in corticosteroid levels is the result of circadian periodicity in the secretion of ACTH (133). Peak 17-OHCS levels at 6:00 AM were associated with an ACTH plasma concentration of the order of 0.25 mU/100 ml, while the low at 6:00 PM corresponded to an ACTH concentration of 0.11 mU/100 ml.

Ungar and Halberg have demonstrated circadian periodic secretions in both adrenals and pituitaries of mice during in vitro studies (185, 186). The magnitude of the adrenal response to added ACTH was greater at 4:00 AM than at 4:00 PM, indicating a time dependency of adrenal sensitivity to a given ACTH dose (185). In addition, pituitaries removed at intervals over a 20 hour period produced a significant rhythmic increase in corticosterone secretion when added to sectioned mouse adrenals in vitro (186). The above findings of the association of corticoid secretion and the periodicity of ACTH release (133), and results describing the functional and predictable periodicity of both adrenal and pituitary secretions (185, 186), infer a more complex control for synchronization of the two cyclical systems.

Pituitary-adrenocortical secretory cycles, while similar in many cases, appear to be species specific as exemplified by diametrical differences in nocturnally active and diurnally active animals. These cycles are reportedly modified only by superimposed changes in the external environment (27).

Orth, Island and Liddle noted in humans that alteration of the individual's normal sleep schedule for a single day had no effect on the 24-hour plasma 17-OHCS cycle (136). However, alterations in the corticoid cycles were effected by introduction of 12-,19-, and 33-hour sleep-wake cycles for the period of at least a week. The dominant

pattern was one of minimal 17-OHCS levels during the early hours of sleep, a rapid, irregular rise during sleep with maximal readings about the time of waking, and an irregular decline to the cyclic low again early in the sleep period. Since this study showed that the plasma corticoid cycle in man could be shortened to 12 hours or lengthened to 33 hours by altering the length of the sleep-wake cycle, and since some individuals on 19- and 33-hour sleep-wake schedules exhibited two 17-OHCS cycles per sleep-wake period, it was concluded that the pituitary-adrenal cycle is not necessarily one of strict 24 hour duration but is also a function of the subject's habitual sleep-wake routine (136).

Evidence indicates that the biological clock regulating these activities is located in the limbic system of the cerebral cortex (57, 69). In addition, the periodicity of ACTH activity is reported to be absent in patients with hypothalamic and temporal lobe diseases, and lesions of the fornix appear to alter adrenocortical rhythmicity (57).

Utilizing a technique of gross brain ablation, Galicich et al. demonstrated corticosterone secretion cycles similar to normal in mice with suprathalamic lesions, thus indicating the dispensability, at least on a short term basis, of the cerebral cortex including the limbic system as part of the control mechanism (55). Suprapontine ablation, on the other hand, including removal of the thalamus and the hypothalamus, resulted in a reduction of rhythmicity of the hypophyseal secretion of ACTH.

Effect of Anesthesia on Peripheral 11-OHCS Concentration

The effect of Nembutal and Surital anesthetics on peripheral plasma 11-OHCS concentration was determined in 28 dogs in 29 experiments, one animal being used in two experiments. The cumulative data for the effect of anesthesia are shown in Figure 3. For comparative purposes, it will be recalled from the previous section that 11-OHCS readings obtained prior to anesthesia gave a mean concentration of 0.26 μg/ml plasma with a standard deviation of 0.07.

All post-anesthesia 11-OHCS values were recorded only under conditions of anesthesia and do not include samples obtained during surgery and during the performance of other experimental procedures. It will be noted that a greater number of points occur within 30-36 minutes following anesthesia and that the number of points diminish thereafter. This is due to the fact that in most cases surgery was begun after that time.

The only attempt to tabulate 11-OHCS levels during surgery is discussed in the section on the effects of hypophysectomy. In all of those cases there did occur a marked rise in peripheral plasma 11-OHCS concentration with the institution of surgical trauma, and this remained at an elevated level throughout the surgical procedure. This elevation

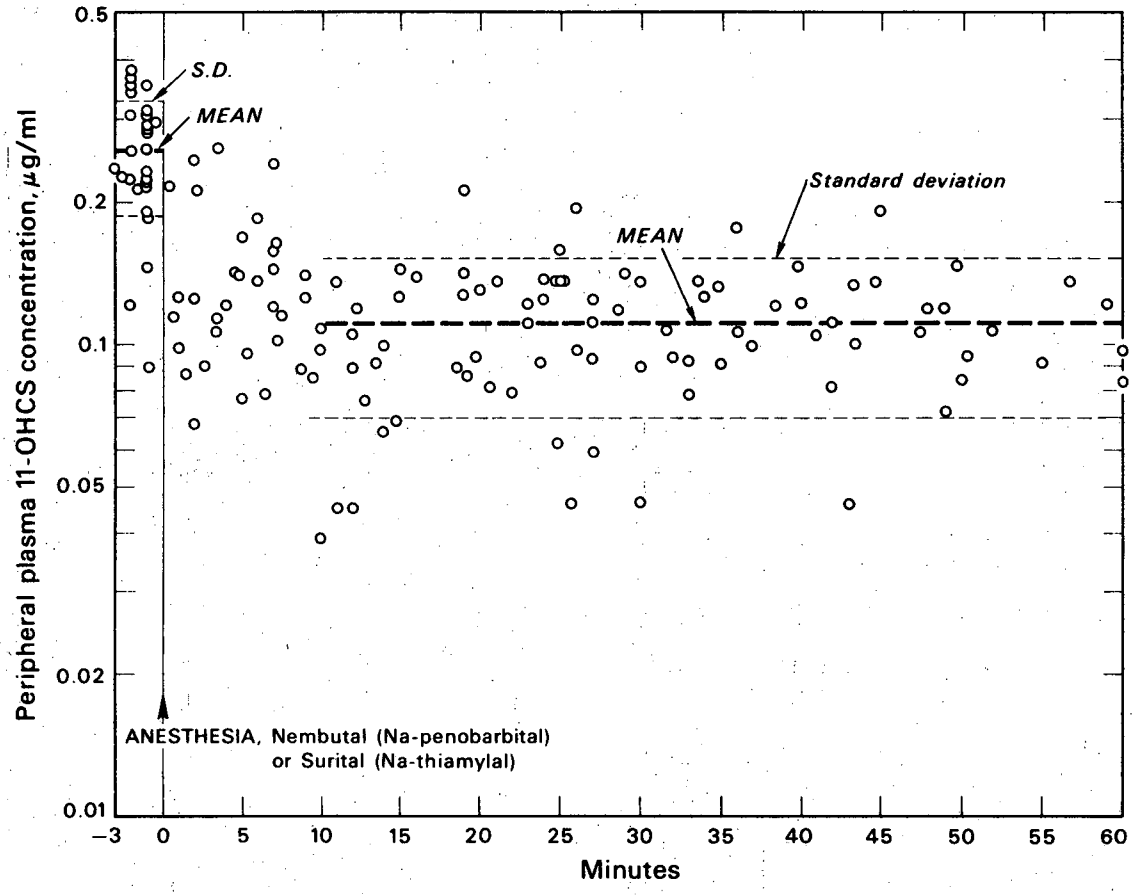

Figure 3. Cumulative peripheral plasma 11-OHCS concentration before and after barbiturate anesthesia (28 dogs).

with surgery appeared to be of the same order of magnitude as the drop in plasma 11-OHCS concentration with anesthesia.

Within a very short period following the administration of anesthesia, 1-3 minutes, the plasma 11-OHCS level fell to the extent that no continuum was apparent between the pre-anesthetic values and those after anesthesia.

As will be appreciated from comparison with the decline of plasma 11-OHCS concentration after hypophysectomy ($T_{1/2}$ = 76 minutes), the suppression of 11-OHCS concentration with barbiturate anesthesia cannot be explained solely on the basis of 11-OHCS metabolism after blocking of ACTH release. Other factors such as fluid shifts from extravascular compartments may be involved. A depression of hematocrit by about 20% was observed following anesthesia; however, this occurred over the period of about an hour. In addition, the concept of a fluid shift associated with anesthesia is complicated by reports of equal distribution of both corticosteroids and ACTH between plasma and lymph (177, 196). An equilibrium distribution of hormones between plasma and other fluids of the extravascular compartment probably also holds true. Plasma protein measurements were not conducted in this study.

By 10 minutes after anesthesia, corticoid concentration fell to what appeared to be a stable level of secretion, and this was maintained

throughout the remaining period of blood sampling. The average 11-OHCS value between 11 and 60 minutes was 0.11 μg/ml plasma, SD 0.04, which was a drop to 43.6% from the pre-anesthetic mean of 0.26 μg/ml.

The findings that pentobarbital and thiamylal anesthetics in dogs cause a rapid depression of 11-OHCS levels are in contrast to the effects of anesthesia observed in humans. Franksson et al. (53) found an elevation in the blood levels of corticosteroids following induction of spinal and Pentothal anesthesia. This rise was attributed by the authors to the stimulating effect of tension, pre-operative preparation and anesthesia.

Sandberg et al. (161), however, found that neither the apprehension of surgery nor the underlying clinical conditions had a significant effect on the elevation of plasma 17-OHCS. The induction of general anesthesia with Pentothal, cyclopropane and ether did cause a rise in the corticosteroid level from a mean of 13 μg/100 ml plasma to an average of 19 μg/100 ml.

Roche, Thorn, and Hills (158) noted no fall in eosinophil levels with the induction of Pentothal anesthesia. One patient showed a drop of 27% following ether anesthesia, and the same patient demonstrated a decline of 75% in the eosinophil level after the administration of 25 mg ACTH. It was postulated that anesthesia alone may block the normal

pituitary-adrenal response and that a maximal "stressful" situation may be necessary to break the inhibition.

Zhukovskii et al. (212) observed a higher level of 17-OHCS in patients with ether-oxygen anesthesia than in those with induced electro-anesthesia. This they attributed to the possibility of fewer disorders in the binding and inactivation of hormones in the livers of patients undergoing electroanesthesia. The fact that the catecholamine level - epinephrine, norepinephrine and dopamine of adrenal medullary and neurosynaptic origin - was higher during electroanesthesia than with ether-oxygen anesthesia suggests a greater activation of the sympatho-adrenal response with the former.

Eik-Nes and Samuels noted an elevation in the 17-OHCS level of mongrel dogs following induction of pentobarbital anesthesia (38). This rise continued from a mean of 5 μg/100 ml, range 2-12, throughout the following 6 hours to 12 μg/100 ml, range 9-15. However, their blood samples showing the rise in corticosteroids were not taken for some time after anesthesia. It was felt that this progressive elevation of cortisol level may have been due not to the effects of anesthesia but rather to the introduction of an intra-arterial cannula for blood sampling. It is of interest as a comparative note, however, that beagles in the present study still showed a depression in circulating 11-OHCS level with introduction of a jugular cannula following anesthesia.

The finding of the present study that barbiturate anesthesia evokes suppression of adrenal steroid secretion supports the pentobarbital-induced depression of free plasma corticoids in rats reported by Rerup and Hedner (155). In addition, Sayers has observed the suppression of corticotropin release with administration of pentobarbital as determined by adrenal ascorbic acid content in rats (164). Ether, on the other hand, caused an elevation in blood ACTH in the latter study, and Hedner measured a 2.3 fold increase of plasma corticosteroids in rats from a control level of 17.6 μg/100 ml, SEM 1.9, to 41.4 μg/100 ml, SEM 2.5, 10 minutes after ether anesthesia (80). The 17-OHCS level after pentobarbital in the latter study was 16.4 μg/100 ml, SEM 2.5.

The pentobarbital induced block of corticotropin release in Rerup and Hedner's investigations was not affected by minor surgical procedures such as skin incision and tail tip cuts in the rat (155). It will be recalled that cannulation of the jugular vein of beagles in the present study after administration of barbituric anesthesia was not sufficiently traumatic to propagate the elevation of circulating 11-OHCS. Major surgical procedures, however, are capable of overcoming this block, as will be discussed in a later section.

The reasons for the discrepancies between the findings of this study in beagles and those reported in rats (155) concerning adrenal steroid suppression, i.e., interference with ACTH release, with

barbituric anesthetics, and the converse observations of corticoid elevation in man are not immediately apparent. It is suggested that the 17-OHCS elevations may have been attributable to such factors as pre-operative preparations and surgical procedures, factors considered by the investigators themselves as possibly promoting high readings. In addition, lack of consistency of other indices of ACTH activity, e. g., no observable fall of eosinophil level with Pentothal in man (158), suggests further questioning of barbituric anesthesia provoked adreno-corticoid elevation.

Dogs anesthetized with barbiturates reportedly respond with a marked rise in corticosteroids with the administration of ACTH (78). This has also been observed in the present study and is discussed in a later section. It has been postulated that barbiturates may depress mediators, possibly hypothalamic, which activate the release of endogenous ACTH (78). In rats subjected to ether anesthesia, however, elevated ACTH levels indicated endogenous and near maximal secretion, for exogenous ACTH had little or no effect on further elevation of corticosteroid levels (166).

Egdahl has demonstrated a fall in adrenal venous cortisol following Nembutal anesthesia in previously cannulated dogs with cerebral decortication (36). The mean values for the decline in these animals were from 10.2 μg/100 ml, SEM 0.8, to 2.1 μg/min, SEM 0.8. Sciatic

nerve stimulation elevated the corticoid level to 14.1 μg/min, and intravenous administration of 25 units of ACTH produced a slightly higher average of 17.8 μg/min, SEM 2.2.

French and co-workers have demonstrated that the principal site of action of Nembutal is the reticular formation (54). The susceptibility of complex interneural systems has been emphasized, and synaptic transmission appears to be blocked long before fiber conduction is affected. The effect of central anesthetics on nerve fiber conduction appears to proceed without reference to the fiber diameter or velocity of fiber conduction.

Slusher and Critchlow have presented evidence in rats that ACTH release is inhibited by higher centers of the central nervous system (172). Gross lesions involving posterior portions of the midbrain and an area at the level of the rostral pons resulted in elevated corticosterone levels, indicating that these structures normally exert an inhibitory effect on the adrenocortical response to stress. Lesions of the posterior diencephalon and the rostral midbrain, on the other hand, resulted in depression of corticosterone to ether stress. The authors concluded that certain areas of the posterior diencephalon and the rostral midbrain must be functional for the normal corticosteroid response to "stress."

Egdahl has demonstrated that dogs with brain removal down to the pons and with neurologically isolated pituitaries show a high "basal"

output of corticosteroids and that nerve stimulation and vena cava constriction result in further elevation of steroids (36). This was presented as evidence for the existence of another center in the hindbrain involved in the control of ACTH release.

According to Egdahl's hypothesis, both the hypothalamus and the hindbrain are capable of releasing neurohumoral substances, corticotropin releasing factor (CRF) and hindbrain factor (HF) respectively, which pass into the circulation and cause the release of ACTH by the pituitary. In the intact animal, the 17-OHCS outputs are basal because of the inhibition by the cerebral cortex and the diencephalon-mesencephalon on the hypothalamus and hindbrain. These inhibitory influences can be overcome by strong afferent stimulation.

Following cerebral decortication, the corticosteroid output was elevated because of removal of reticular formation inhibition and constant activity of the hypothalamus in the release of CRF (36). The hindbrain center remained inhibited, and following anesthesia the steroid output fell because of the blocking action on the reticular formation. Following afferent nerve stimulation, the corticosteroid output in both intact and decorticated anesthetized animals rose because the ACTH-releasing centers were intact.

In dogs with the brain removed down to the pons and with isolated pituitaries, the 17-OHCS output was elevated because of removal of

inhibition by the diencephalon-mesencephalon on the hindbrain. ACTH release may have taken place either because of the spontaneous release of HF or because of tonic afferent impulses. Nembutal anesthesia in Egdahl's animals had no effect on the depression of corticosteroid levels because of its primary action on the reticular formation with little or no influence on most hindbrain centers or on peripheral nerve fiber transmission.

Because of normal nerve response following anesthesia, it is unlikely that pentobarbital has a direct effect on the pituitary (36). In addition, failure to produce depression of post-anesthetic "resting" 17-OHCS output levels in dogs with brain removal is evidence against the direct action of Nembutal on the adrenals.

Effect of Hypophysectomy on Peripheral Plasma 11-OHCS Concentration

The cumulative data for peripheral plasma 11-OHCS concentration before and after hypophysectomy are shown in Figure 4. This figure represents information from 25 dogs, 8 of which contributed only one 11-OHCS reading (7 during the pre-hypophysectomy period and one post-hypophysectomy). Only concentration values obtained within 20 minutes prior to hypophysectomy were included in that group. Following hypophysectomy only those values which were not influenced by further surgical manipulations and experimental techniques were included.

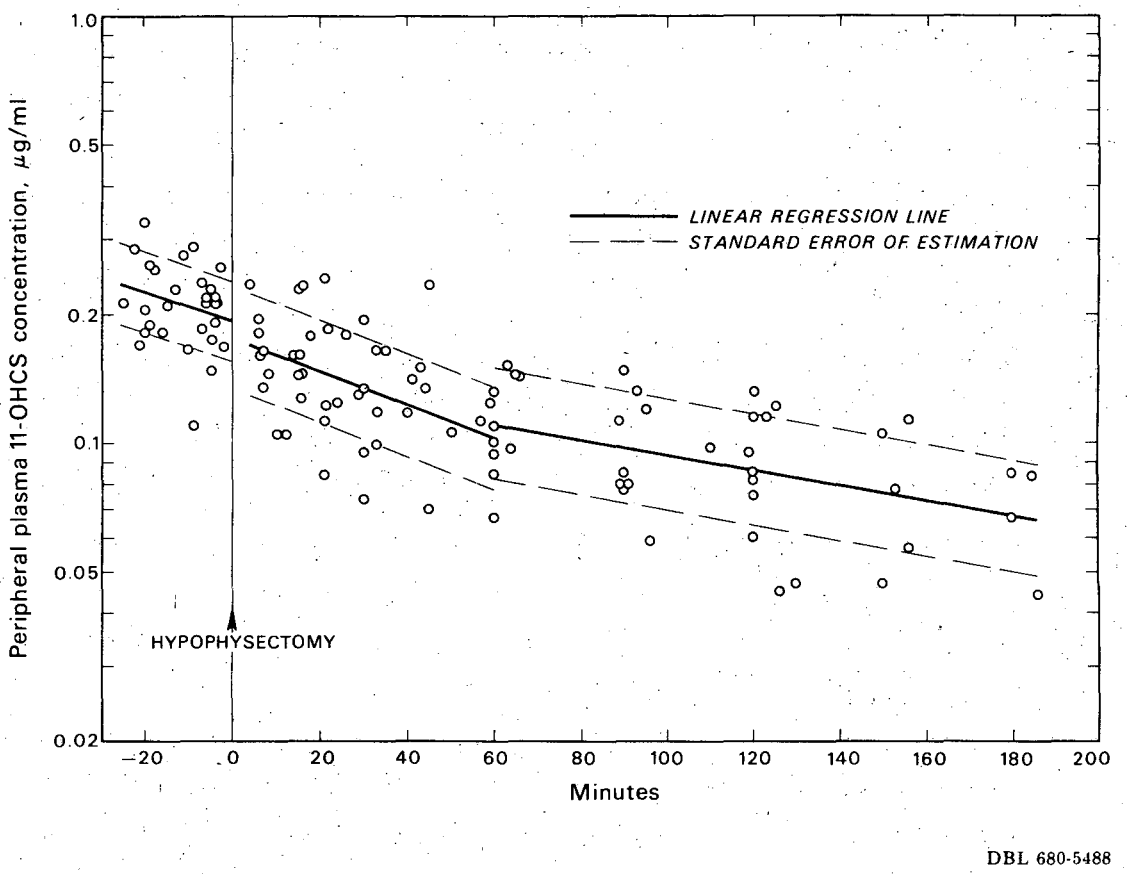

Figure 4. Cumulative peripheral plasma 11-OHCS concentration before and after hypophysectomy (25 dogs).

The mean for the 11-OHCS concentrations during the pre-hypophysectomy period was 0.21 μg/ml, SD 0.05. These values represent the 11-OHCS picture not in the normal "resting" animal but in the anesthetized state immediately following surgery and closure of the incision.

The linear regression determination prior to anesthesia indicates a declining slope over the 20 minute period. This decline may indicate a "recovery" or return of the elevated 11-OHCS resulting from operative trauma levels to a more "basal" concentration. Computation of this slope[1] was determined by the method of linear regression of distributed points assuming a linear relationship of these points with time. The program for determination of the slope also computes 11-OHCS values on the slope of best fit for any given time as well as the 95% confidence limits for location of the regression line.

From construction of the regression line, the peripheral 11-OHCS concentration at 20 minutes pre-hypophysectomy was 0.23 μg/ml, SEE limits 0.18-0.28. At the time of hypophysectomy the 11-OHCS concentration had fallen to 0.19 μg/ml, SEE limits 0.16-0.24.

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[1] The units for slope values in this section are in terms of plasma 11-OHCS concentration per unit time, or $\frac{\mu g/ml}{min}$.

The level of corticotropin release and the response of the adrenal cortex prior to hypophysectomy, from the standpoint of elevated corticosteroid levels under the conditions studied, must be considered as resulting from the trauma of surgery. In the use of untrained animals many activities may constitute a form of "stress" resulting in the output of corticosteroids above that in the normal "resting" animal. The term "stress" applies here to the more specific hypophyseal-adrenal response of elevated ACTH and corticosteroid levels rather than "the sum of all non-specific changes caused by function or damage" (168). Sharp increase in the free corticosterone of rat plasma has been noted from such unsuspected activity as movement of the animal cages from one location to another (67).

The observations of Eik-Nes and Samuels should be mentioned again, however, in that frequent removal of blood in trained unanesthetized dogs over a 6 hour period did not instigate changes in peripheral 17-OHCS levels (38). The same authors demonstrated increased adrenocortical activity within 15 minutes following arterial cannulation in anesthetized animals. Anesthetized dogs in the same study subjected to arterial cannulation, to heat, to cold, and to intravenous injections of bacterial polysaccharides exhibited mean plasma 17-OHCS levels of 7, 12, 24, and 25 μg/100 ml respectively as compared with the control value of 3 μg/100 ml in trained dogs. In man following pentobarbital

anesthesia and during surgery, the 17-OHCS level was found to rise gradually to approximately 3 times normal, and it was concluded that the magnification of the steroid level was proportional to both the duration and to the severity of surgery (161).

Hume and Nelson noted elevations in the corticosteroids in adrenal venous blood of dogs during operative trauma (88). The morning following surgery, the levels dropped, and with the infusion of ACTH into the unanesthetized animals, the 17-OHCS output rose to the high levels observed during surgery. Hilton et al. concluded that the blood of non-hypophysectomized dogs contained sufficiently high levels of ACTH after surgery to induce high and possibly maximal corticosteroid secretion (84). In man a rise from a mean 17-OHCS level of 5.6 μg/100 ml before surgery to 31.4 μg/100 ml following surgery was noted (53). Extreme individual variations in the maximal adrenocortical secretion capabilities in both dogs and man have been emphasized (12, 17, 78). These individual differences among dogs appear to reflect the adrenal secretory capacity rather than the rate of ACTH release (78).

Although the causal relationship is uncertain, the circulating level of eosinophils in man appears to be intimately related to pituitary-adrenocortical activity, whereby ACTH stimulation of the adrenal cortex evokes a fall in the eosinophil level (158). The eosinophil count doubled within the first half hour following operative incision in man in all cases

in which a comparison was available and then fell abruptly to 86 to 100% that of normal. The pattern of eosinophil decline with surgery appeared to be analogous in shape and time to that after injection of ACTH (158). It was suggested that this pattern with surgery was initiated by increased release of the patient's own ACTH which in turn influenced the elevation of corticosteroid secretion.

In man, dogs and rats the disappearance of corticosteroids from the circulation follows an exponential pattern; that is, 17-OHCS are removed from the circulation at a rate proportional to the concentration present (15, 16, 38, 62, 119, 152, 208). These observations have come largely from study of the disappearance of intravenously injected corticosteroids.

The slope for 11-OHCS clearance in Figure 4 was computed from the linear regression of post-hypophysectomy 11-OHCS values assuming an exponential clearance. The range of maximal and minimal slopes for the cumulative 11-OHCS values was determined from the 95% confidence limits for location of the regression line.

The slope for the 11-OHCS concentrations between 4 and 60 minutes following hypophysectomy was -0.009, which is representative of a half-life for 11-OHCS of 76 minutes. The 95% confidence limits for location of the regression line gave a $T_{1/2}$ range of 52-150 minutes. The slopes of best fit for the 11-OHCS values from 60-120 minutes and

120-186 minutes were quite similar, -0.0031 and -0.0037 respectively, suggesting a common component of corticosteroid degradation throughout the 126 minute time period. The slope of best fit for the 11-OHCS decline between 60 and 186 minutes was -0.004, which indicated a $T_{1/2}$ for 11-OHCS of about 170 minutes.

The two different slopes illustrated in Figure 4 indicate metabolism of 11-OHCS in a two compartment system: one represented by a $T_{1/2}$ of 76 minutes for corticoids in the main circulatory volume and the other becoming evident after 60 minutes with a $T_{1/2}$ of 170 minutes. The latter component probably accounts for metabolism of 11-OHCS migrating from the extravascular and intracellular compartments. In conjunction with this concept, Stark et al. noted that the concentrations of corticoids in plasma from the femoral artery and in lymph from the thoracic duct were comparable in anesthetized dogs, and with the administration of ACTH, the corticoid rise was parallel in the plasma and lymph (177). Ligation of the efferent adrenal veins promoted a greater than 8 fold increase in lymph corticoid concentration from a mean of 15.8 μg/100 ml, SD 10.4, before ligation to 137.5 μg/100 ml, SD 83.1, after ligation. These findings demonstrate that lymph functions not only as a major hormone transport system but also as a vehicle by which these hormones are returned to the circulation at a relatively slow rate. The kinetics of a two-compartment concept of corticosteroid

disappearance has been presented by Yates and Urquhart and is considered later in the discussion of adrenal corticoid metabolism (208).

Eik-Nes and Samuels observed the disappearance of 17-OHCS in dogs under varying conditions of "stress" following the intravenous injection of cortisol (38). In conscious animals accustomed to venipuncture the half-life for cortisol was 150 minutes. The cortisol clearance was slower in anesthetized animals subjected to cannulation, cold, heat and injected pyrogens. Approximation from these authors' data showed the $T_{1/2}$ of cortisol in anesthetized-cannulated dogs was about 4 hours and 40 minutes.

Sandberg et al. suggested that the cortisol clearance picture in animals subjected to severe traumatic situations is comparable to that seen in dying human patients (160). It was noted that elevated 17-OHCS levels were invariably present in moribund patients within 72 hours of expiration, and the same consistency of high levels was also seen in surgical patients in which life threatening situations developed, e.g., hemorrhage and shock. In less critical situations, however, the development of elevated corticosteroid levels appeared to be dependent on the site and length of surgery and on the anesthesia (161, 184).

In man the mean clearance half-time of labeled cortisol has been found to range between 60 and 116 minutes (15, 16, 57, 208). Peterson and Wyngaarden found a mean $T_{1/2}$ of 83 minutes, range 62-138, in one

study (142), while in another, Peterson et al. found the mean biological half-life for hydrocortisone in normal humans to be 114 minutes, range 90-130 minutes (143). With the infusion of hydrocortisone in quantities ranging from 50 to 500 mg, even though plasma levels rose to between 70 and 600 μg/100 ml, the clearance half-times for individual subjects were essentially identical (143). This would indicate that the rate of metabolism of cortisol is proportional to the concentration over quite a wide range of concentration levels.

Plager et al. in observations in man found half-lives for injected cortisol of 72, 114, and 86 minutes respectively in samples obtained from the femoral artery, antecubital vein and hepatic vein (150). These differences with respect to sampling site, they suggest, represent different factors involved in the turnover rates of the steroid. Without further evidence, however, this concept is questionable when considering the variability for corticoid half-times presented by different investigators, in addition to the effect of the time of day in sampling.

While the metabolism of corticosteroids forms an important area in the consideration of adrenal steroid decline following hypophysectomy, this is not, as such, a part of the problem under consideration. Therefore, a discussion of this aspect has been deferred and is included in the Appendices.

Effect of Hypophysectomy on Adrenal Venous 11-OHCS

Adrenal 11-OHCS Concentration

Concentration values for adrenal venous plasma 11-OHCS before and after hypophysectomy were obtained from 24 dogs, of which 4 contributed only pre-hypophysectomy readings. Figure 5 shows a single experiment as a representative example of the effect of hypophysectomy on peripheral and adrenal venous plasma 11-OHCS concentration.

The peripheral 11-OHCS concentration was about 0.106 μg/ml at the time of hypophysectomy and fell to 0.074 μg/ml after 30 minutes. This decline was somewhat faster than that seen in most other individual cases and in the cumulative picture discussed later. The fall of 11-OHCS after 30 minutes became less steep and reached a level of about 0.035 μg/ml at 5 hours.

The pre-hypophysectomy adrenal plasma 11-OHCS reading was 3.06 μg/ml at -9 minutes. Shortly after hypophysectomy, 3-5 minutes, the 11-OHCS level fell dramatically, to 0.21 μg/ml by 21 minutes. The decline of adrenal corticoids after hypophysectomy is known to be exponential (38, 67), and in this case, the 11-OHCS concentration appeared to fall logarithmically between 9 and 18 minutes. In addition, the fall of corticoids in the blood of hypophysectomized dogs with the

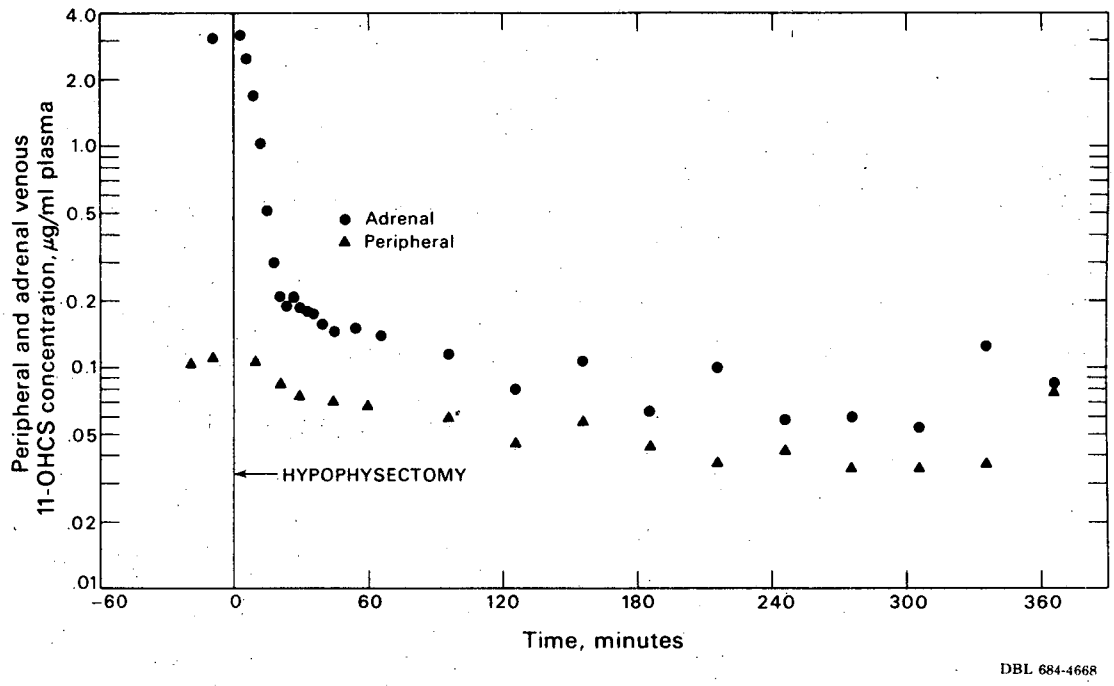

Figure 5. Peripheral and adrenal venous plasma 11-OHCS concentration before and after hypophysectomy.

interruption of ACTH infusion approximates a first-order exponential decay (161).

The linear regression line of best fit for adrenal steroid values between 9 and 18 minutes gave a slope[1] of -0.198 and a $T_{1/2}$ for ACTH of 3.5 minutes with 95% confidence limits of 3.2 and 3.8 minutes. The half-life of ACTH as determined from the decline of adrenal venous plasma 11-OHCS concentration is slightly influenced by readings of recirculating adrenal steroids. When the peripheral 11-OHCS concentration values were subtracted from the adrenal values and a linear regression line was determined, a slope of -0.228 was obtained with a $T_{1/2}$ for ACTH of 3.0 minutes. When the adrenal blood flow was stable, as it was in this case, subtraction of peripheral 11-OHCS values from adrenal readings determined the adrenal 11-OHCS output.

Fluorescence of plasma samples obtained after 3 to 4 hours post-hypophysectomy was probably the result of several factors: 1). Adrenal corticoid secretion resulting from minute amounts of residual ACTH in the blood and ACTH diffusing from the extravascular "outer pool," 2). 11-OHCS still resident in the circulation (11-OHCS $T_{1/2}$ = 76 min) and 11-OHCS diffusing from the extravascular "outer pool," 3). 11-OHCS

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[1] The units for slopes in this section are $\frac{\mu g \text{ 11-OHCS/ml plasma}}{\text{minute}}$.

from adrenal "leakage," and 4). fluorescence of non-steroid origin as reported by others, which is reportedly a linear function with the amount of plasma used (26, 51, 80). Variable amounts of non-specific fluorogens equivalent to 0.03 μg/ml have been reported (110), and residual fluorescent material which produced readings up to 0.07 μg/ml has been found in plasma of adrenalectomized and hypophysectomized animals (14).

Based on the fluorescence of cortisol as 100%, Rudd and Black recently reported 0-6% fluorescence with equivalent amounts of pregnanetriol and pregnanetriolone in low concentrations and fluorescence of 62% and 15% for the two compounds respectively but at higher levels (159). While these steroid by-products are present only in very small amounts in body fluids under normal conditions, background fluorescence might be considerably influenced by combined fluorescence from the products of steroid metabolism.

Fluorescence inherent in readings of adrenal 11-OHCS concentrations after 240 minutes, as shown in Figure 5, was estimated to be equivalent to 0.06 μg/ml from values. As determined from peripheral 11-OHCS values, this fluorescence would be equivalent to about 0.04 μg/ml. When the higher background value was subtracted from adrenal 11-OHCS readings, a slope of -0.218 was determined between 9 and 18 minutes giving an ACTH half-life of 3.2 minutes, see Figure 6. The

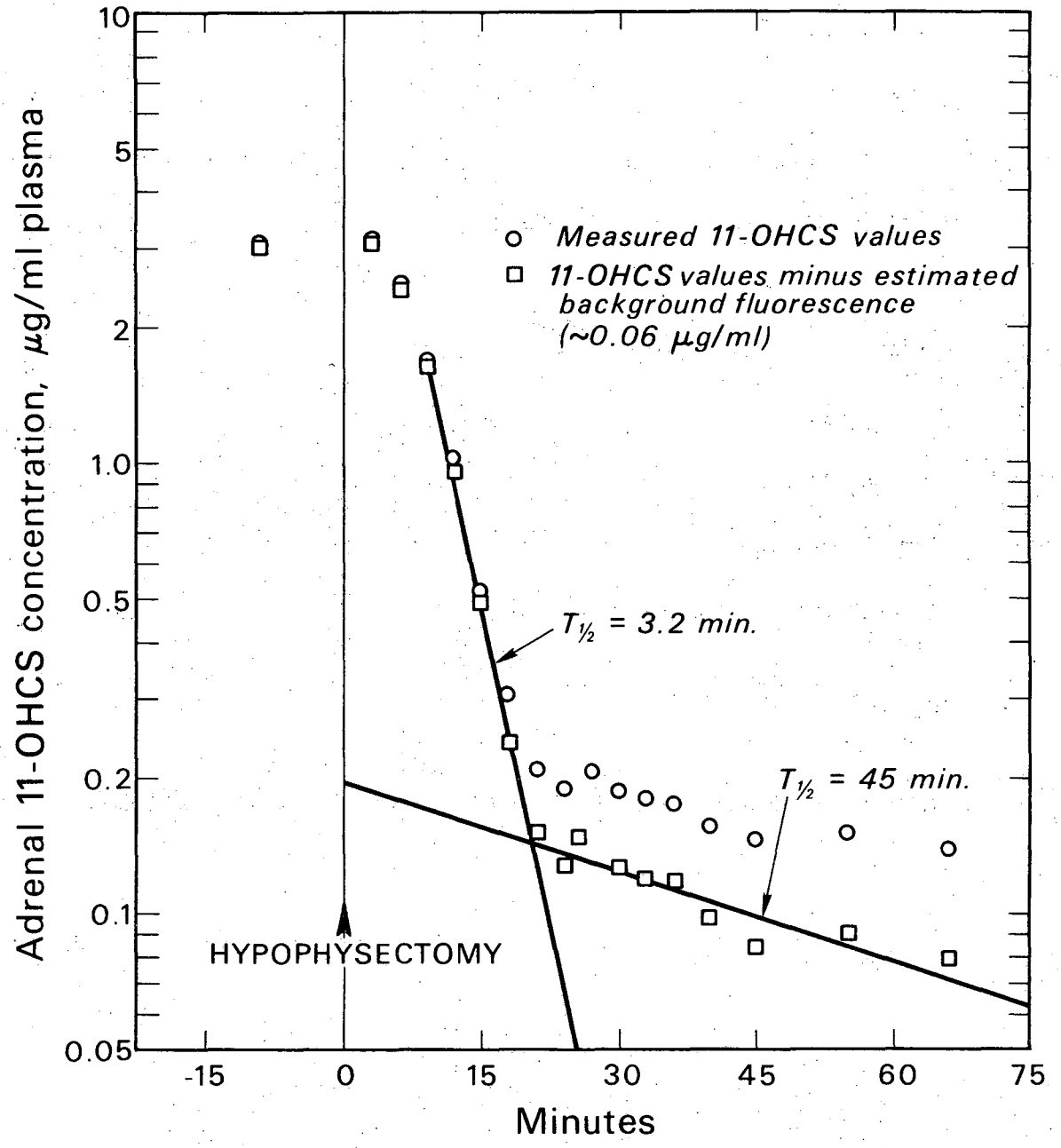

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Figure 6. Adrenal venous plasma 11-OHCS concentration before and after hypophysectomy, subtraction of background fluorescence.

second, shallower component of the curve, between 21 and 66 minutes, determined a $T_{1/2}$ of 45 minutes from a slope of -0.014.

The second component of the curve in Figure 6 representing a slower metabolism of ACTH is probably the result of diffusion of the pituitary hormone into the circulation from the extravascular "outer pool." While the existing data do not warrant extensive speculation, this extravascular content of ACTH at a relatively low concentration may in actuality represent a rather large amount of physiologically active ACTH distributed throughout the tissues of the body.

It will be noted in Figure 5 that while the adrenal 11-OHCS concentration after hypophysectomy approached that of the peripheral concentration, comparable values were not attained even after 5 hours. Similar findings were noted in other experiments, and in no case did the levels become equal. The slopes for the adrenal and peripheral 11-OHCS readings between 186 and 306 minutes were -0.003 and -0.002 respectively. The concentration difference between adrenal and peripheral 11-OHCS levels at 180 minutes was 0.03 μg/ml and that at 300 minutes was 0.02 μg/ml. The adrenal 11-OHCS level, while slowly approaching that of the peripheral concentration, was significantly higher throughout the 2-hour interval as determined by the respective standard errors of estimation. It is suggested that the higher adrenal 11-OHCS values were due to low level adrenal stimulation from ACTH diffusing from the "outer

pool," corticosteroid "leakage" from the adrenal, and weak adrenocortical stimulation from non-hypophyseal sources.

The similarity of an ACTH half-life derived from 11-OHCS levels in adrenal venous whole blood to the $T_{1/2}$ determined in plasma is illustrated in Figure 7. The pre-hypophysectomy 11-OHCS value at -3 minutes was 5.3 μg/ml. The linear regression line for values between 4 and 14 minutes gave a half-life for ACTH of 3.4 minutes with a 95% confidence limit range of 3.0 to 3.8 minutes.

The cumulative data of adrenal venous plasma 11-OHCS concentration before and after hypophysectomy in 24 dogs are shown in Figure 8. The mean pre-hypophysectomy 11-OHCS concentration between -20 minutes and the time of hypophysectomy was 6.11 μg/ml, SD 2.38. The slope of the initial 11-OHCS decline after hypophysectomy was -0.138 which indicated a $T_{1/2}$ for ACTH of 5 minutes, 95% confidence limit range of 3.6 to 7.6 minutes. A slope of -0.001 determined for plasma steroid values between 35 and 90 minutes gave a $T_{1/2}$ of 5.4 hours.

Concentration values based on the regression line of best fit for the recirculating peripheral plasma 11-OHCS (see, Figure 4) were subtracted from those of the adrenal concentration between 4 and 20 minutes. The resulting slope was -0.158 with concentrations of 4.43 μg/ml at 4 minutes and 0.35 μg/ml at 20 minutes post-hypophysectomy. This slope is indicative of an ACTH half-life of 4.4 minutes.

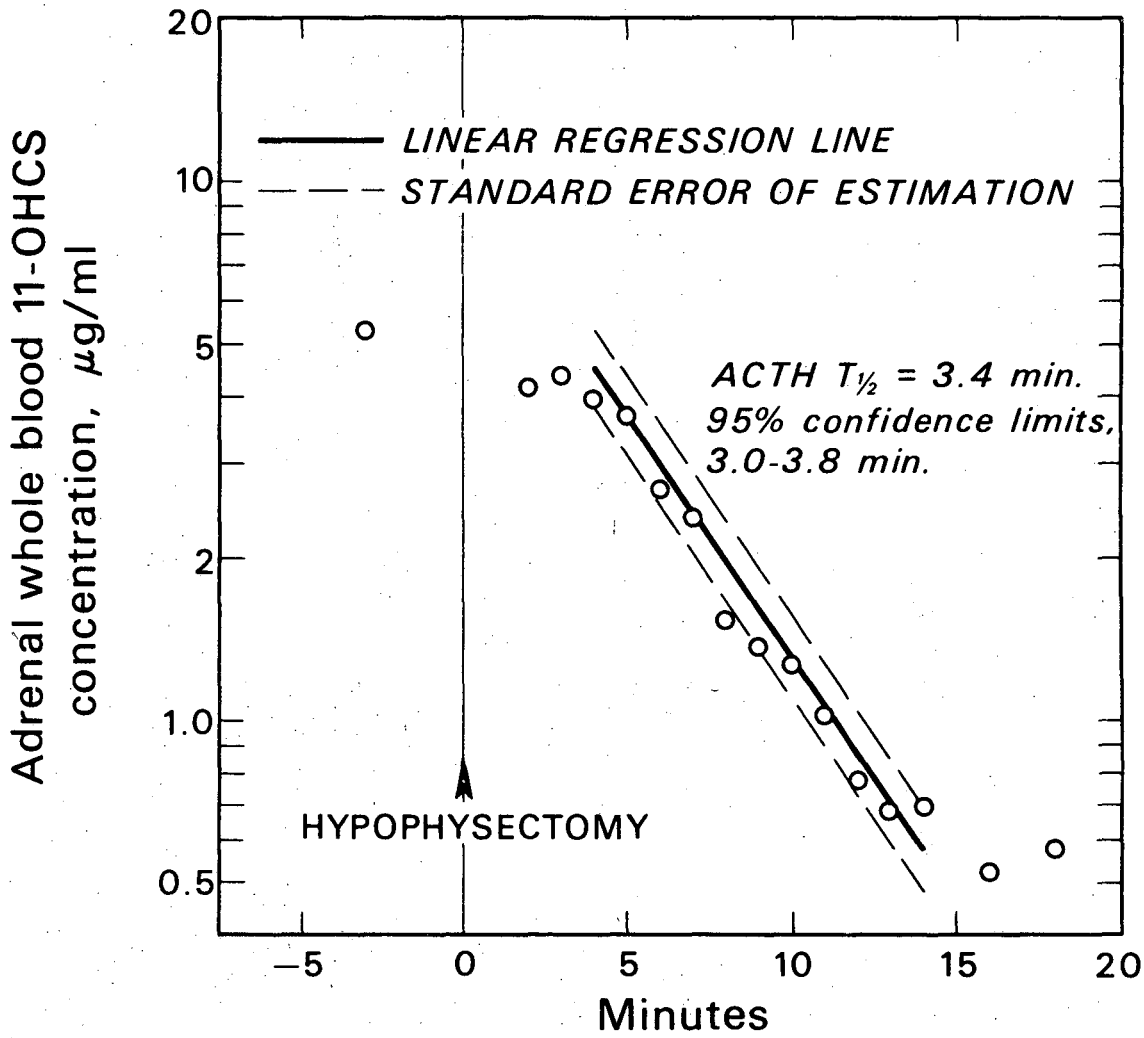

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Figure 7. Adrenal whole blood 11-OHCS concentration before and after hypophysectomy.

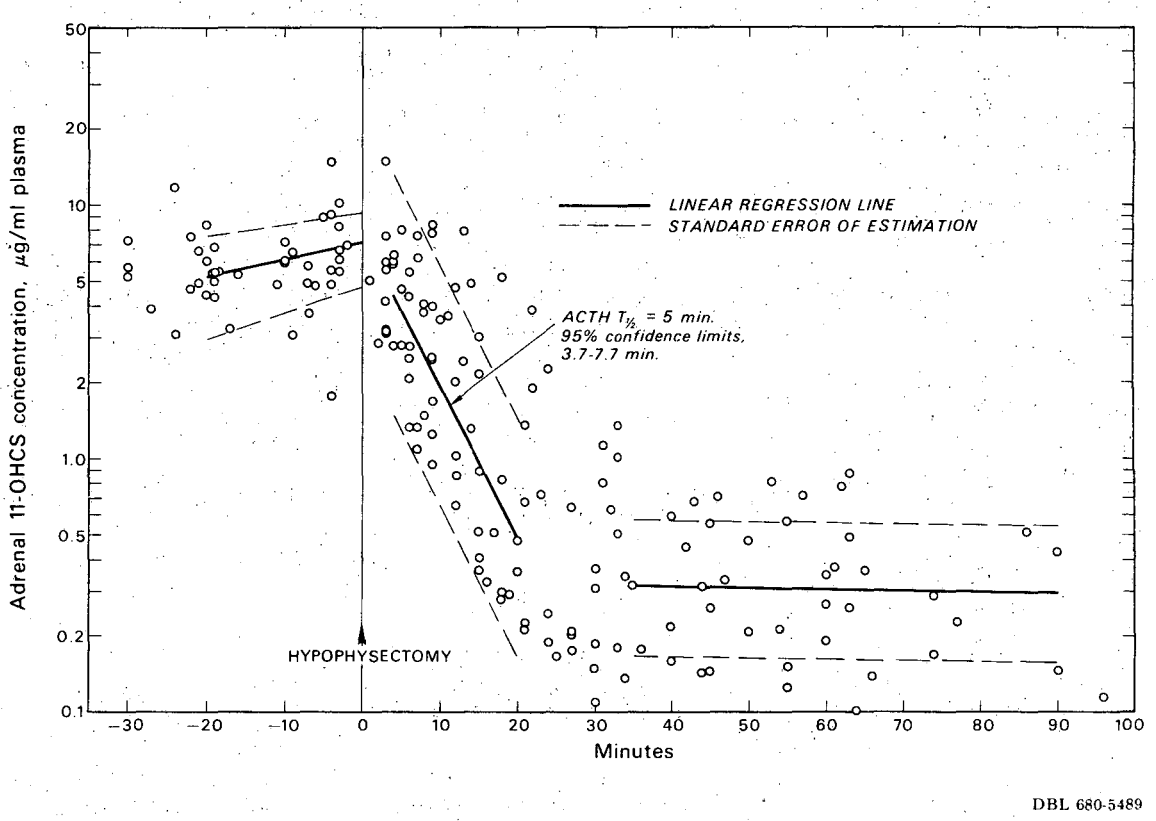

Figure 8. Cumulative adrenal venous plasma 11-OHCS concentration before and after hypophysectomy (24 dogs).

Due to the wide distribution of steroid concentrations after hypophysectomy, the individual experiments were re-examined, and it was concluded that these could be grouped according to two general criteria: I) those animals which demonstrated an initial rapid fall in 11-OHCS concentration within a short period of time, 3 - 7 minutes, after hypophysectomy; and II) those which showed a decline after a more "prolonged" period, 8 - 15 minutes, following hypophysectomy. Eight animals were included in group I and five in group II. Excluded from these groups were animals which contributed only pre-hypophysectomy values, those which did not contribute to the cumulative slope determination between 4 and 20 minutes, and those which showed irregularities during the pre- or post-hypophysectomy periods.

Figure 9A shows the 11-OHCS concentration values for animals in group I with a fall in 11-OHCS "shortly" after hypophysectomy. The slope of -0.183 for 11-OHCS values between 4 and 15 minutes in Figure 9A was representative of a half-life for ACTH of 3.8 minutes with a range of 2.8 to 5.5 minutes as determined from 95% confidence for location of the regression line. A slower rate of 11-OHCS decline was indicated at about 15 minutes at which time the 11-OHCS concentration had dropped approximately 93% from that at the time of hypophysectomy. By 25 minutes the 11-OHCS level had fallen to about 0.2 μg/min. When the regression line for the 11-OHCS values of adrenal plasma

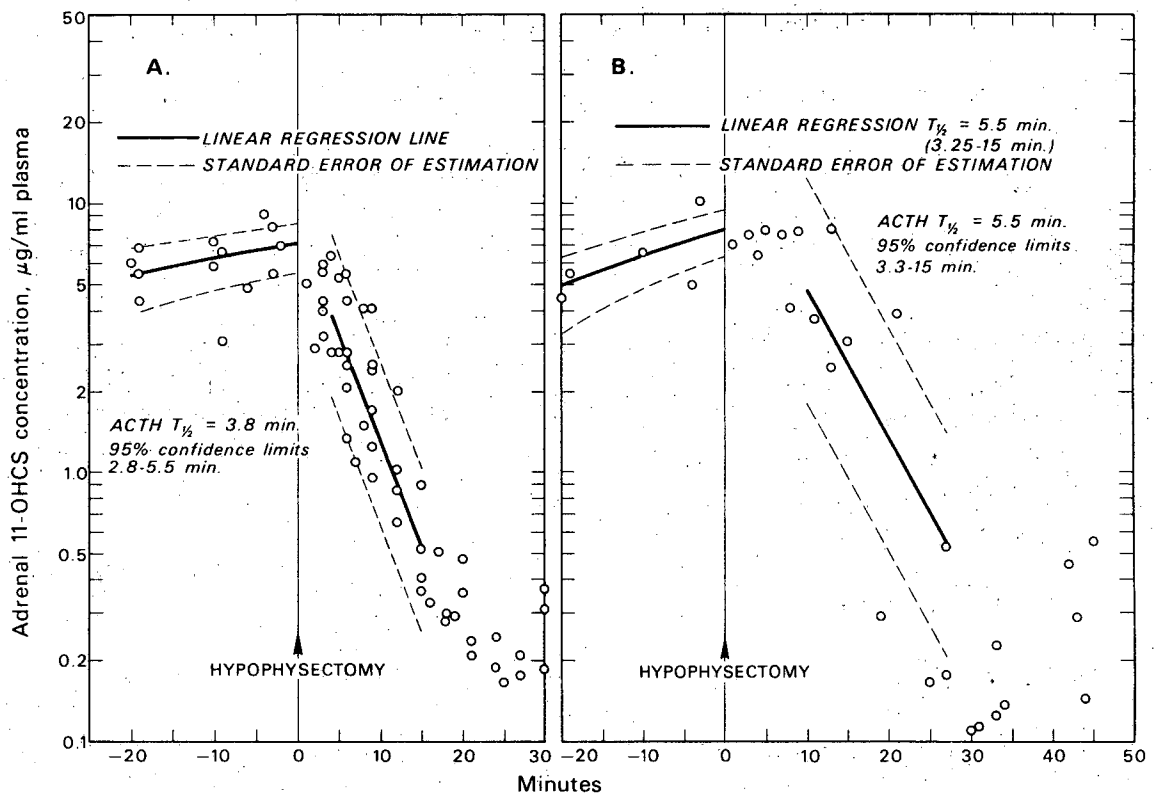

Figure 9. Cumulative adrenal venous plasma 11-OHCS concentration before and after hypophysectomy. A. 11-OHCS decline 3-7 minutes after hypophysectomy. B. 11-OHCS decline 8-15 minutes after hypophysectomy.

minus peripheral plasma concentrations was determined, a slope of -0.21 and a $T_{1/2}$ for ACTH of 3.3 minutes were obtained.

Adrenal plasma concentration values for animals in group II, which showed a fall of 11-OHCS after a latent period following hypophysectomy, are presented in Figure 9B. The mean of the 20 minute pre-hypophysectomy values was 6.39 μg/ml, SD 1.88. The regression line between 10 and 27 minutes after hypophysectomy gave a slope of -0.129 with a half-life for ACTH of 5.5 minutes and a 95% confidence limit range of 3.3 to 15 minutes. With subtraction of peripheral plasma concentration values between 10 and 27 minutes, a slope of -0.143 was obtained indicating a $T_{1/2}$ for ACTH of 4.8 minutes.

The reason for the discrepency between values in groups I and II for the time of fall of 11-OHCS concentration after hypophysectomy is not immediately apparent. Both exhibited rapid $T_{1/2}$ values for ACTH, 3.3 and 4.75 minutes respectively. However, the wide 95% confidence limit range of group II, the greater number of values represented in the 11-OHCS decline and the narrower range of distribution in group I tend to place a greater reliance on the determination of 3.3 minutes in group I as the more nearly accurate estimation of the half-life of ACTH. Both groups demonstrated low 11-OHCS levels following the rapid exponential decline, indicating the absence of endogenous ACTH (53, 88, 158). It is possible that the latent period

before 11-OHCS decline for animals in group II may be the result of time for high ACTH levels to fall below those producing maximal adrenocortical stimulation.

The slopes of the linear regression lines, the equivalent half-lives for ACTH and the 95% confidence limit ranges for the effect of hypophysectomy on adrenal venous plasma 11-OHCS concentration in this section are listed in Table I. The $T_{1/2}$ values for ACTH determined in the groups listed fall within a relatively narrow time interval of 3.2 to 5.5 minutes. While each determination contributed to a further clarification of the corticotropin degradation pattern, emphasis should be placed on the $T_{1/2}$ of 3.3 minutes as most representative of ACTH inactivation, from the discussion above.

Adrenal 11-OHCS Output

In this study the adrenocortical 11-OHCS output is expressed as measured from a single gland in units of micrograms 11-OHCS/minute/10 kilograms body weight. Ten kilograms was chosen as a convenient reference comparison since most dogs used were of about that weight. Corticosteroids measured from adrenal effluent blood in a variety of animals have been variably expressed as micrograms/minute (55, 61, 88, 187, 201), as micrograms/10 minutes, micrograms/hour, micrograms/kilogram/24 hours (18, 52, 115), as

TABLE I

ACTH HALF-LIFE VALUES DERIVED FROM THE DECLINE OF ADRENAL PLASMA 11-OHCS CONCENTRATION AFTER HYPOPHYSECTOMY

11-OHCS Values Included in the Determination	Slope	ACTH $T_{1/2}$ (minutes)	95% Confidence Limit Range for $T_{1/2}$ (minutes)
Total cumulative adrenal 11-OHCS concentration	-0.138	5.0	3.6 - 7.6
Cumulative adrenal 11-OHCS minus peripheral 11-OHCS	-0.158	4.4	
Group I: 11-OHCS decline within 3-7 minutes	-0.183	3.8	2.8 - 5.5
Group I minus peripheral 11-OHCS	-0.209	3.3	
Group II: 11-OHCS decline within 8-15 minutes	-0.129	5.5	3.3 - 15
Group II minus peripheral 11-OHCS	-0.143	4.8	
D-192: Representative experiment (minus background)	-0.218	3.2	2.9 - 3.5
D-196: Whole blood 11-OHCS	-0.205	3.4	3.0 - 3.8

micrograms/minute/100 grams (62, 192), as micrograms/hour/gland (78) and as micrograms/adrenal/hour/kilogram body weight (87).

The cumulative data for the effect of hypophysectomy on adrenal 11-OHCS output are shown in Figure 10. These values were obtained from the same 24 animals as in the previous section. The average of the pre-hypophysectomy readings between -20 and zero minutes was 9.62 μg/min/10 kg, SD 3.52. A linear regression determination gave only a slightly ascending slope[1] of 0.006 with an intercept at the time of hypophysectomy of 9.69 μg/min/10 kg, SEE limits 13.21 - 6.17.

The wide distribution of points following hypophysectomy makes analysis of the results very difficult. It was apparent by 3 to 5 minutes after hypophysectomy that 11-OHCS output had begun to fall very sharply and that by 15 minutes some of the values had reached low points between 0.1 and 0.2 μg/min/10 kg. It is equally apparent, however, that in some cases the steroid output was maintained at the surgical, pre-hypophysectomy level up to 10-15 minutes following hypophysectomy and that the fall in output did not become obvious until after 15 minutes.

It was found that the data from Figure 10 could be separated according to dogs which exhibited an 11-OHCS output decline between

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[1] The units for slopes in this section are $\mu g/min^2/10$ kg.

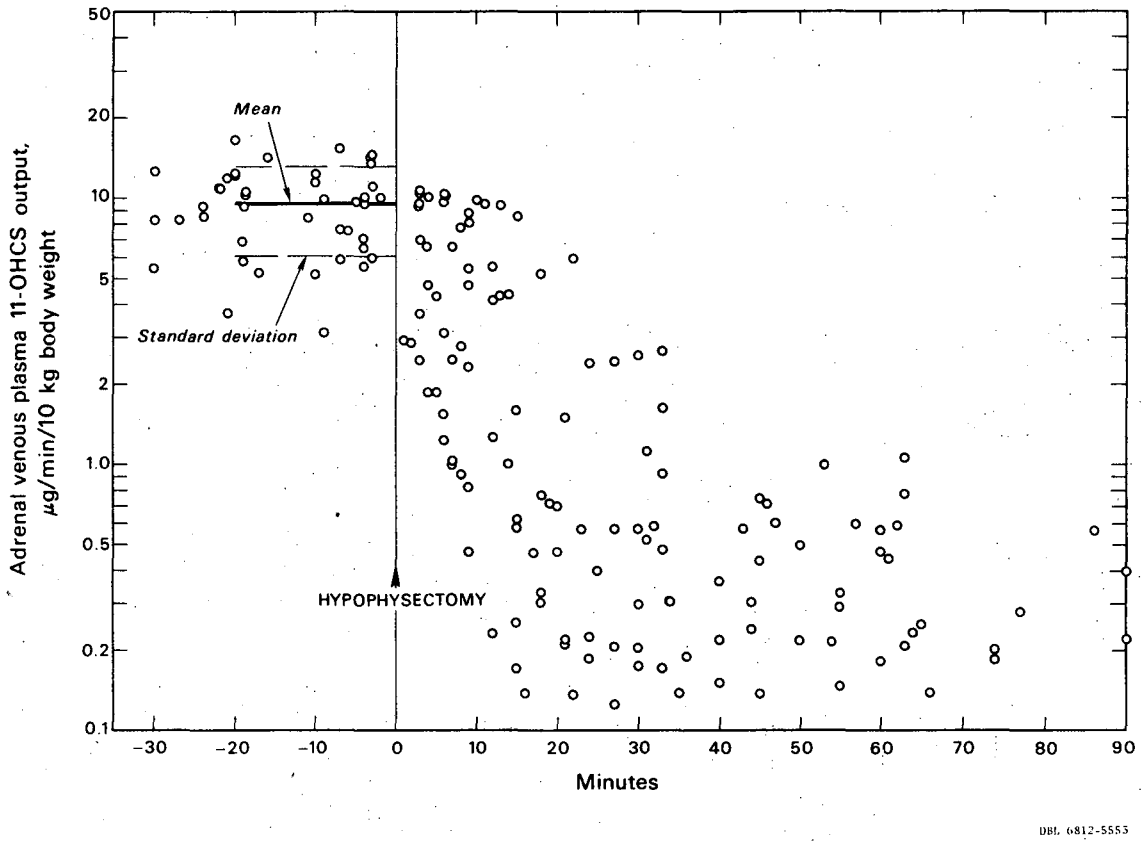

Figure 10. Cumulative adrenal 11-OHCS output before and after hypophysectomy (24 dogs).

3 and 7 minutes after hypophysectomy, group IA, and those in which the output did not begin to fall until 8 to 15 minutes. The cumulative data for the respective groups are shown in Figures 11A and 11B.

The mean pre-hypophysectomy 11-OHCS output between -20 minutes and the time of hypophysectomy for group IA, Figure 11A, was 9.05 μg/min/10 kg, SD 3.00. Between 3 and 6 minutes after hypophysectomy 11-OHCS output began to fall precipitously and continued to decline until about 15 minutes. After that time the fall became less steep and by 25 to 30 minutes had reached a level of about 0.2 μg/min/10 kg. The linear regression calculation for the output values between 3 and 15 minutes gave a slope of -0.204, which is representative of an ACTH half-time of 3.5 minutes with a 95% confidence range of 2.5 to 5.5 minutes.

Figure 11C shows the adrenal 11-OHCS output for animals in group IA in terms of mean values of successive time intervals. The estimated $T_{1/2}$ for ACTH from the steepest corticoid decline between about 8 and 15 minutes was 2.5 minutes. 11-OHCS readings from one dog with a low initial post-hypophysectomy output level were not included in the mean determinations because of absence of pre-hypophysectomy 11-OHCS values. The estimated ACTH half-life for that animal was 3.7 minutes.

The mean pre-hypophysectomy 11-OHCS output for animals in group IIB, Figure 11B, was 9.91 μg/min/10 kg, SD 3.06, and after

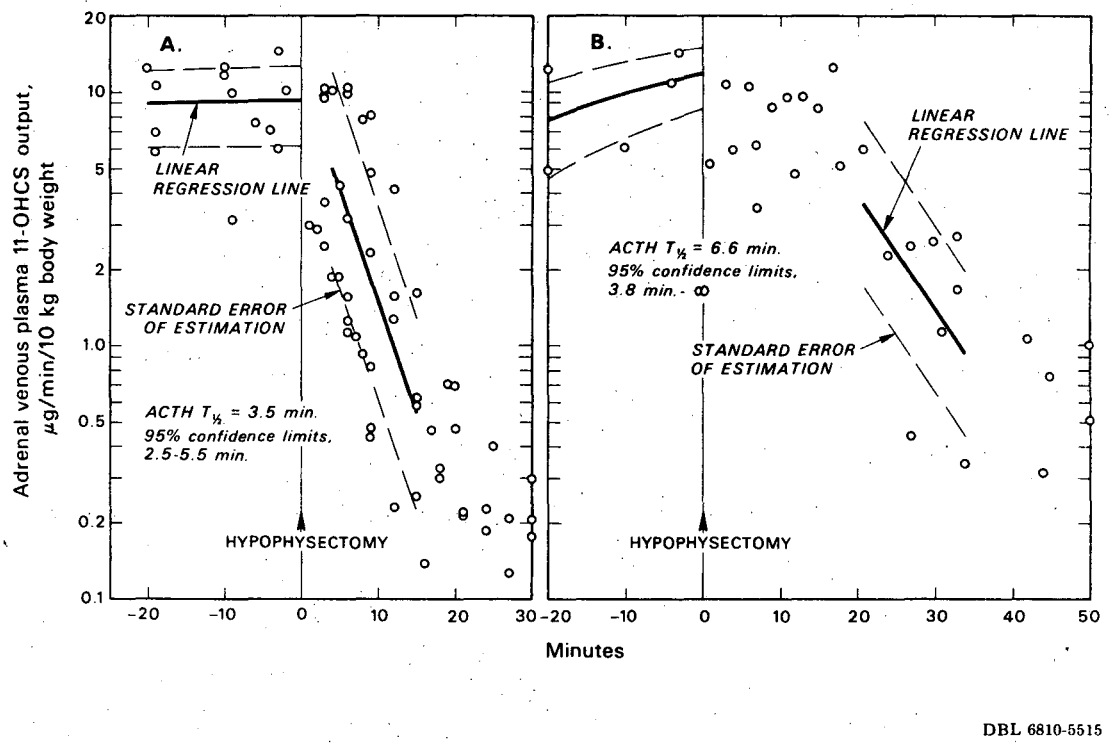

Figure 11. Cumulative adrenal 11-OHCS output before and after hypophysectomy. A. 11-OHCS decline 3-7 minutes after hypophysectomy. B. 11-OHCS decline 8-15 minutes after hypophysectomy.

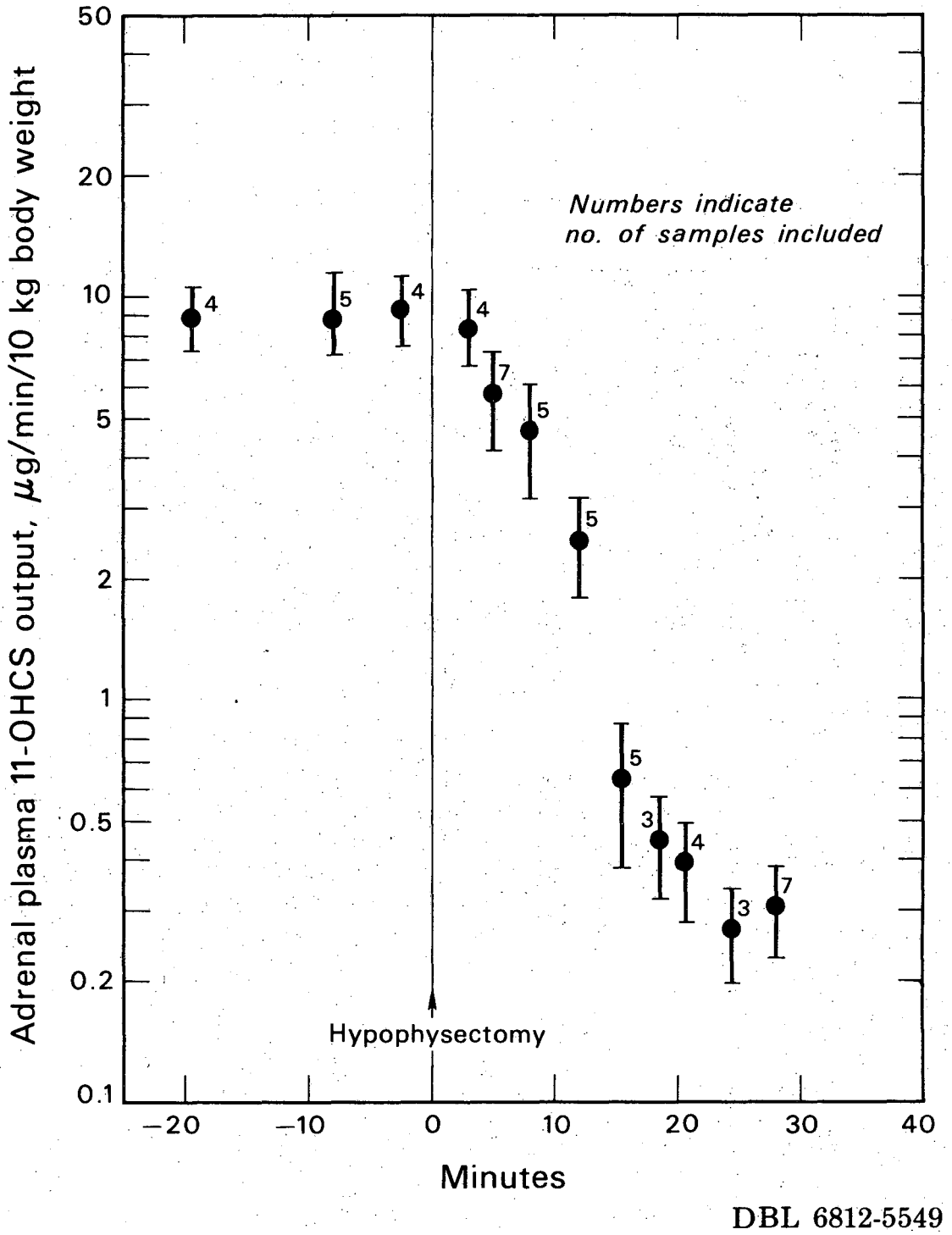

Figure 11C. Mean adrenal 11-OHCS output for successive 3-5 minute intervals before and after hypophysectomy. Values were derived from dogs demonstrating 11-OHCS decline 3-7 minutes after hypophysectomy (Figure 11A). Bars denote standard error.

hypophysectomy the widely distributed output values remained high for about 20 minutes. The linear regression line of the 11-OHCS values between 21 and 34 minutes gave a relatively broad slope of -0.103 and a $T_{1/2}$ for ACTH of 6.6 minutes, 95% confidence limit range between 3.75 minutes and infinity.

It was noted that, after hypophysectomy, three of the animals of this group exhibited an increase in adrenal blood flow by as much as 25-30%. In those cases the slopes of best fit determined from 11-OHCS concentration (Fig. 9B) and from 11-OHCS output (Fig. 11B) might not show the parallel relationship they would if the flow rates were constant.

Obviously, with such a confidence range, 3.75 minutes to infinity, little credence can be placed on the half-life of ACTH as determined from those output values. In addition, since the 11-OHCS output did not drop to the lows noted for dogs in group IA, the possibility of incomplete hypophysectomy exists. The fact that the $T_{1/2}$ of 6.6 minutes determined for group IIA lies well outside the 95% confidence limit range of that for group IA, 2.2 to 5.5 minutes, adds further question to the validity of these data.

It is suggested from observations in this study that the characteristics of the disappearance of ACTH from the circulation of beagles as determined by adrenal 11-OHCS output include the following: (1) the

initial drop in adrenal 11-OHCS output resulting from the interrupted influence of endogenous ACTH began within a short time after excision of the pituitary; (2) the disappearance of ACTH from the circulation follows an exponential pattern which exhibited a half-life of 3.5 minutes, determined from adrenal plasma 11-OHCS output, with a confidence limit range between 2.5 and 5.5 minutes, and (3) after 15 minutes, the fall in ACTH level became less steep and by 25 minutes had reached a level to produce an adrenal 11-OHCS output of 0.2 μg/min/10 kg.

It should be emphasized that inspection of the sella turcica in hypophysectomized dogs for determination of residual pituitary tissue was not attempted. Rather, completeness of hypophysectomy throughout this study was based on low adrenal plasma 11-OHCS measurements after hypophysectomy. This basis was derived from findings of Ganong and Hume (58) and Hume and Nelson (88) that adrenal 17-OHCS output in hypophysectomized dogs was usually near zero but always less than one μg/min, from recordings of adrenal 17-OHCS output of 0.2 μg/min in hypophysectomized dogs by Wise et al. (201), and from findings of Urquhart et al. that an adrenal cortisol secretion rate of 0.24 ± 0.6 μg/min denotes absence of endogenous ACTH (191).

It was at first thought that an appropriate criterion for hypophysectomy would be the exponential fall of 11-OHCS within 3-7 minutes

as observed for the animals in groups I and IA. However, one dog included in groups II and IIA, which showed a steroid decline after a more "prolonged" period, demonstrated a very fast half-time for ACTH and a very low 11-OHCS measurement after the exponential drop.

As shown in Figure 12 for this dog, both the drop in adrenal venous plasma 11-OHCS concentration and 11-OHCS output gave a $T_{1/2}$ for ACTH of about 3.3 minutes. The lowest 11-OHCS readings were at 63 minutes: 0.21 μg/ml for the concentration and 0.26 μg/min/10 kg for the output, after which a definite decline in 11-OHCS was still apparent.

Even though the fall of ACTH and the low 11-OHCS values for the dog in Figure 12 were more in fitting with animals with complete hypophysectomy, the time interval between hypophysectomy and the decline of 11-OHCS was considerably longer in the former case. In view of this evidence, the characteristics of ACTH kinetics after hypophysectomy cannot include the restriction of a 3 to 7 minute time interval before detectable depression of the hormone activity. It is possible that the extended latent period may have been the result of temporary incomplete hypophysectomy and the time for removal of residual fragments and/or the result of unusually high levels of corticotropin release due to mechanical trauma to the pituitary just prior to

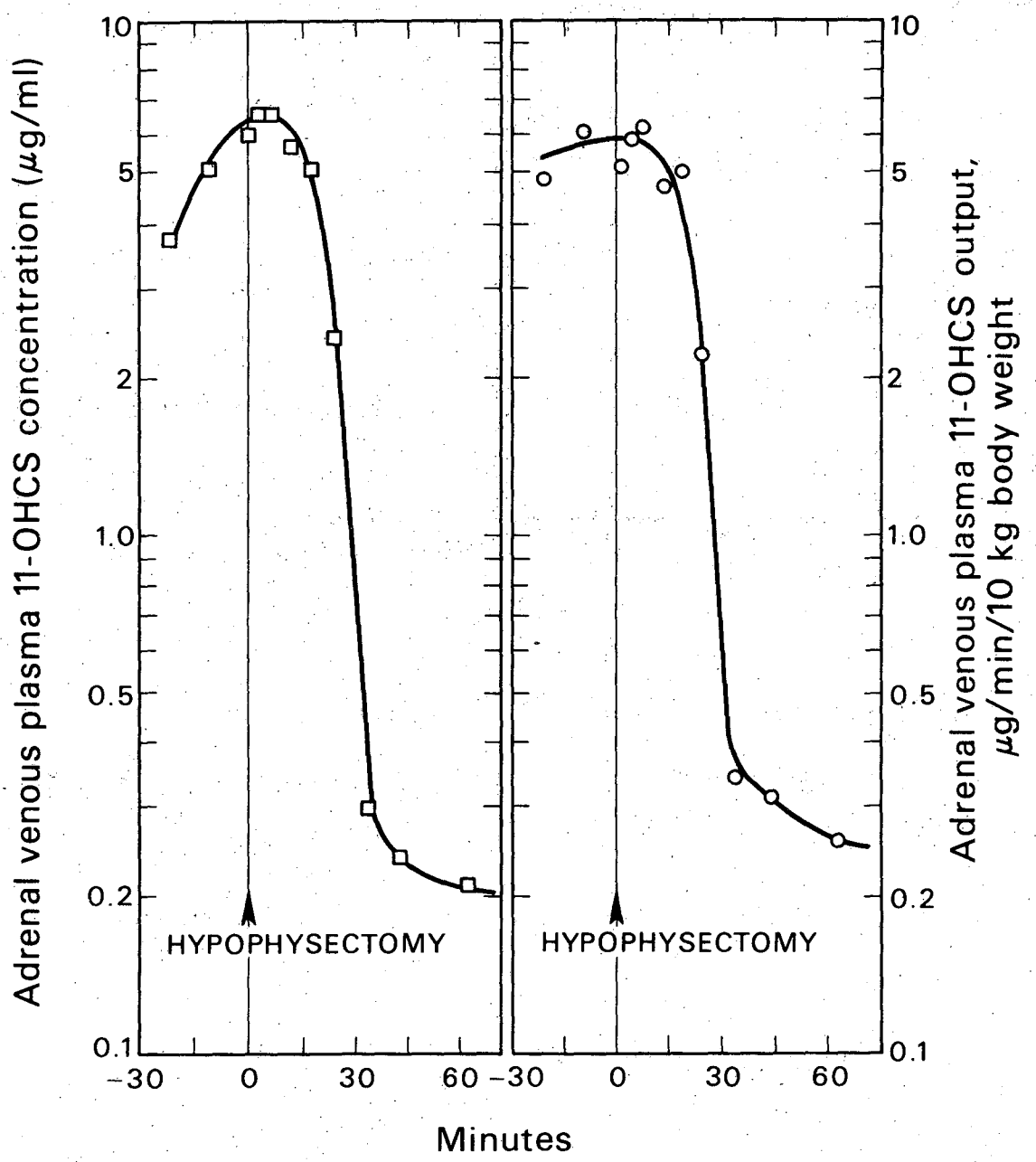

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Figure 12. Adrenal venous 11-OHCS before and after hypophysectomy: Example of prolonged latent period after complete hypophysectomy.

hypophysectomy. The basis for this latent period would appear to be of considerable interest and of possible significance, but further speculation would require more than the data presented.

A sampling of adrenal corticoid output values in dogs from the literature as well as those from this study is listed in Table II.

Measurement of adrenal 17-OHCS secretion and adrenal ascorbic acid depletion determinations have shown that ACTH has a very short biological half-life. Following intravenous injection of ACTH into intact rats, Greenspan, Li and Evans noted with the ascorbic acid depletion method that ACTH disappeared in a logarithmic fashion with a half-life of 5.5 minutes (64). From adrenal weight changes in parabiotic rats, a technique cited as quantitatively questionable (181), Van Dyke et al. determined the average half-life of ACTH to be approximately 17 minutes (196). The "adrenocorticotropic hormone space" of those animals was determined to be 43 percent of the body weight with concentration of the hormone essentially equal between the plasma and lymph. Syndor and Sayers found the half-life of endogenous ACTH following hypophysectomy in previously adrenalectomized rats to be about 1 minute as indicated by the ascorbic acid depletion method (175, 181): 0.95 minutes was determined in one experiment and 1.25 in another (181). Seventy-five percent of the activity disappeared within 3 minutes. In the same experiment no loss of biological activity of ACTH

TABLE II

LISTED ADRENAL CORTICOID OUTPUT VALUES IN DOGS

Authors & Reference No.	Corticoid Output	Conditions of Measurement
Wise, Brunt & Ganong (201)	10.8 μg/min, SD 2.0 B Wts 7.5 - 16.8 kg	Pentobarbital anesthesia, adrenal cannulation and craniotomy control (dura opening only)
Hume & Nelson (88)	12.7 μg/min, range 9 - 22.2 B Wts 14 - 26 kg	Ether anesthesia and surgery
Ganong & Hume (57, 58)	7.2 μg/min, SEM 0.8 7.2 μg/min, SEM 0.2 5.9 μg/min, SEM 0.8 B Wts 10 - 15 kg	Ether anesthesia and surgery " " " " " " " "
This Study	9.6 μg 11-OHCS/min/10 kg, SD 3.5	Barbiturate anesthesia and surgery

was noted when blood was incubated in vitro at 37°C for one minute, suggesting that the rapid inactivation did not occur directly in whole blood.

Urquhart cited a time constant of 5 to 7 minutes for ACTH in the blood of acutely hypophysectomized dogs, as determined from the infusion of ACTH (189). Although the methodology was admittedly imprecise, a distribution space for ACTH comparable with that of the plasma volume was proposed. The determination of an ACTH half-life of 3.5 minutes in the present study indicates a faster rate of hormone inactivation in dogs than has been previously estimated.

Early estimations using the ascorbic acid depletion technique gave a half-life for porcine ACTH in man of 5 to 15 minutes (41). A later study of Meakin et al. using the hypophysectomized dog assay method, gave a comparable range of 4 to 18 minutes for the disappearance of commercial ACTH in man (114). When the logarithm of ACTH activity was plotted against time in Meakin's study, it appeared that there were two rates of ACTH decline, a rapid initial rate and a slower second component. This two component concept of ACTH inactivation is in keeping with findings of equal distribution of ACTH between blood and lymph (196) and with evidence in the present study of a rapid and then a slower rate of corticotropin disappearance after hypophysectomy.

As described previously, low corticoid values in the range of 0.1 to 0.24 μg/min are considered as indications of complete

hypophysectomy and absence of endogenous ACTH (56, 88, 191, 201). Complete removal of the pituitary and of hypophyseal fragments is necessary for the insurance of depressed 17-OHCS to those low values. Zukowski and Ney compared the 17-OHCS output values of both peripheral and adrenal venous blood of control and pituitary stalk-sectioned dogs and found them to be similar (213). There was reportedly no significant difference in the elevation of 17-OHCS in the two groups after exogenous ACTH administration.

In those animals the pituitary stalk was sectioned just below the diaphragm sellae and a sheet of aluminum foil was placed across the area of transection to prevent regeneration of portal vessels (213). Most stalk-sectioned dogs responded with elevated 17-OHCS output in response when subjected to laparotomy, traction on the sciatic nerve and administration of metyrapone (metopirone), an inhibitor of corticoid biosynthesis. Those animals not responding to metyrapone showed normal responsiveness to ACTH, indicating sufficient baseline ACTH to maintain normal adrenocortical response, but depletion of pituitary ACTH "reserve" as a result of stalk section. The mechanism of elevated ACTH response to trauma in those conditions is uncertain, but a systemic circulatory connection transporting corticotropin-releasing factors from the hypothalamus was postulated along with the suggestion of an independent influence of hypothalamic regulation.

Further evidence has been presented by Jackson, Egdahl and Hume that even after prolonged periods of time, pituitary stalk section does not abolish the release of ACTH in response to specific trauma (90). While pituitary stalk section leads to reduced adrenal responsiveness to ACTH, definitive elevation of 17-OHCS output in chronically (1-8 months) stalk-sectioned dogs was observed after sciatic nerve stimulation, aortic constriction and ACTH administration. The corticoid output attained, however, remained intermediate between normal basal output and that after normal maximal ACTH administration.

In birds, pituitary stalk transection or autotransplantation of the adenohypophysis caused complete and permanent gonadal atrophy but only mild depression in the synthesis and release of TSH and ACTH (126). It has been proposed that, in chickens, there may exist an extra-hypophyseal source of ACTH or ACTH-like substance which is capable of stimulating adrenal secretion of corticosterone for up to 50 days following hypophysectomy (126).

As emphasized by Harris, "Few procedures have given such discordant results as those involving pituitary stalk section in studies of anterior pituitary function" (71). Investigators cited above have shown evidence for continuance of a certain degree of pituitary function after stalk section, while Harris has pointed to posterior pituitary

atrophy and indefinite cessation of glandular activity with severence of the neural supply (71). There may be some significance in this reference to posterior pituitary atrophy only. Reports of hypophyseal function following stalk section have been attributed by Harris to rapid regeneration of hypothalamic portal vessels across the site of transection (71). It is difficult to correlate this with evidence of pituitary-adrenal function, though somewhat reduced, in stalk-sectioned dogs in which portal vessel regeneration was blocked (213).

Harris has stated that "a search of the literature has failed to reveal any reliable data indicating normal secretion of A. C. T. H. . . . under basal or stress conditions in the absence of hypophyseal portal vessels connecting the median eminence and anterior pituitary gland" (72). Yet Zukowski and Neys' stalk-sectioned dogs upon microscopic examination showed no portal vessel regeneration while exhibiting reduced pituitary activity (213). Variable degrees of necrotic anterior pituitary tissue were seen, but viable tissue was also present. In addition, Thomson and Zuckerman have reported that with pituitary stalk section and absence of portal vessel regeneration, ferrets still showed light-induced estrous response (183). This pituitary-gonadal dependence was based on the conclusion that vessels of the pituitary stalk form part of a neurohumoral reflex pathway between the retinal receptors and the anterior pituitary.

In view of the conflicting evidence, and considering reports of ACTH secretion from pituitary tissue transplanted at sites remote from the sella turcica and other reports of abolished secretion under similar conditions (71, 72, 126), it is painfully clear that a definitive concept relating the pituitary stalk to hypophyseal secretion awaits further evidence and insight.

With graded hypophysectomy in dogs, i. e., increasingly complete pituitary removal, Ganong and Hume have demonstrated that the sequence of loss of endocrine function is gonad - thyroid - adrenal (58). Removal of up to three-quarters of the anterior pituitary resulted in no detectable effect on endocrine function, while 97-99 percent hypophysectomy resulted in complete atrophy of all three target tissues. With the retention of as little as 5-25 percent of the anterior lobe, gonadal and some gonad-thyroid insufficiencies were apparent, but isolated thyroid and adrenal insufficiencies were not observed. Very small remnants of anterior pituitary tissue are capable of stimulating the adrenal to some degree of function in spite of a considerable atrophy of the latter. This has been evidenced by increased contralateral adrenal weight after unilateral adrenalectomy, increased peripheral 17-OHCS level during immobilization stress, and adrenal corticoid output levels above those following hypophysectomy (58).

In studies of graded hypophysectomy in patients with metastatic cancer, Van Buren and Bergenstal observed marked depression of the

urinary gonadotropin level despite resurgence of thyroid and adrenal function in an instance in which approximately 29% of the pituitary remained (193). With 10% or less of the pituitary remaining, the patients were permanently corticosteroid dependent and were judged to have complete hypophysectomies by "endocrinological evaluation." It is of interest that after hypophysectomy residual fragments did not degenerate despite loss of blood supply from the pituitary stalk, suggesting a sufficient blood supply to the pituitary from the dural covering (193).

Effect of Hypophysectomy on Adrenal Blood Flow

Throughout this study, the procedures were periodically plagued, and the interpretation of results often complicated, by unexpected and unexplained decreases and even complete interruption of adrenal blood flow. Adrenal blood flow as affected by hypophysectomy was recorded in the 12 dogs for which ACTH half-lives were determined, and the data are presented in Figure 13. Flow was measured from the volume per minute adrenal effluent of a single gland, and each value in the graph represents the mean of samples collected during successive 5-minute periods.

The mean values show a surgical adrenal blood flow of 3.3 ml/min, SEM 0.4, for a single gland between 15 and 20 minutes before

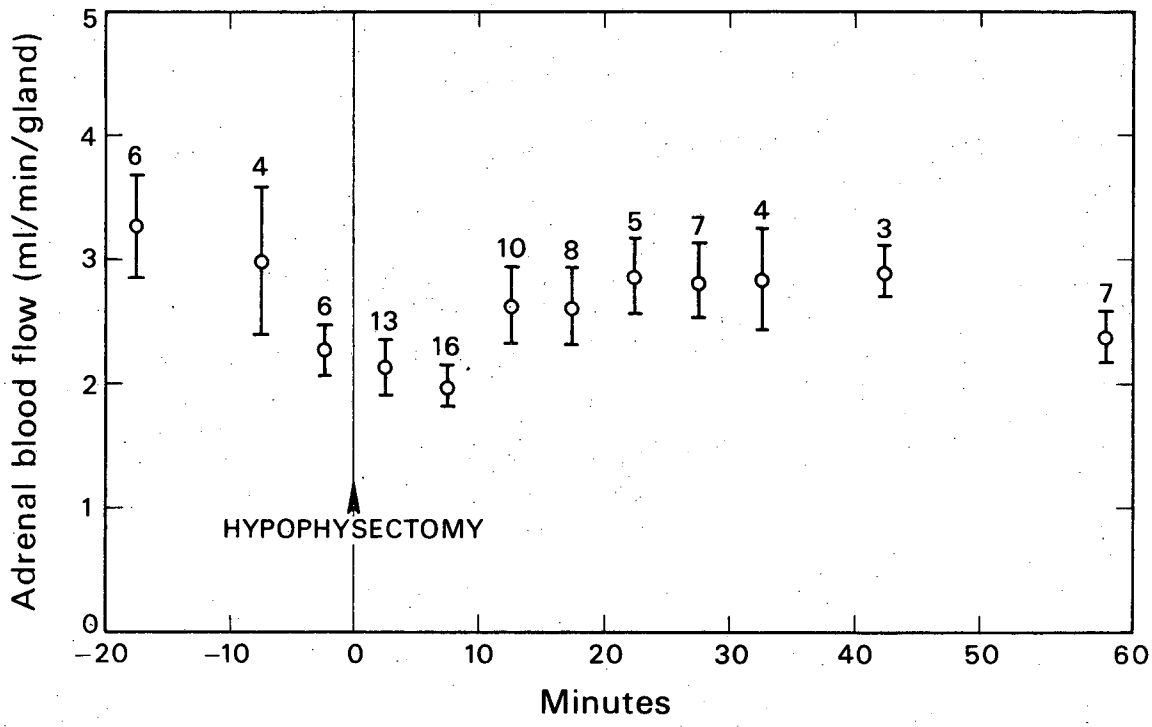

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Figure 13. Adrenal blood flow before and after hypophysectomy. Vertical bars denote standard error of the mean, and numbers at the apices indicate the number of samples included.

hypophysectomy. The mean flow then showed a significant decline from 5 minutes before until about 10 minutes following hypophysectomy, after which the flow level rose to between 2.6 and 2.8 ml/min. However, this decline in adrenal flow was probably not the result of hypophysectomy as judged by the low levels prior to pituitary excision. It is possible that the depression may have been induced by trauma or mechanical stimulation during lumboadrenal vein cannulation. The data presented here indicate that adrenal blood flow is not significantly affected by hypophysectomy.

These findings are in keeping with those of Monos, Koltay and Kovach, who demonstrated that in spite of a decrease in femoral arterial pressure after hypophysectomy in dogs, adrenal blood flow was unchanged and in some cases increased (122). After 60 minutes, the femoral arterial pressure had decreased an estimated 30%, while no significant change was recorded in mean blood flow of the abdominal aorta. A 15-20% greater decrease in adrenal vascular resistance than that in the abdominal organs and the posterior part of the body was recorded.

Adrenal blood flow in dogs has been measured by Hartman, Brownell and Liu who found no definite relationship between flow and animal weight (73). This was true of animals within a given weight group, but significant differences occurred between the mean flows of animals in 5-10 kg and 10-15 kg groups, the latter flow rates being lower.

That adrenal corticosteroid output remains stable over a wide range of variation in adrenal blood flow has been suggested by a number of investigators. Nelson and Hume, in their description of ACTH assay in hypophysectomized dogs, stated that wide variations in blood flow can occur without appreciably affecting the assay (130). Similar relationships were observed in their surgical shock studies (88). This is in agreement with the findings of Hilton and co-workers, that in perfused dog adrenals in situ, the rate of hydrocortisone secretion did not appear to be related to changes in adrenal blood flow (84). Hechter et al. were unable to determine an apparent relationship between the rates of total or individual steroid secretion and the rate of adrenal venous flow in anesthetized dogs (78).

Frank et al. measured adrenal blood flow and corticosteroid output in dogs prior to stress of hemorrhagic shock, during shock, and in response to ACTH given during shock (52). At adrenal flow rates above 17% of pre-shock values, the corticosteroid secretion rates were 60-100% that of the pre-shock levels. At lower flow rates, below 17% of control, a sharp decline in corticoid secretion to 40% or less of control rates was observed.

When hemorrhagic shock was superimposed on operative trauma, adrenal blood flow in dogs was markedly reduced, but the minute output of corticoids increased as witnessed by the considerable rise in steroid

concentration in the blood (89). With blood replacement, corticoid secretion returned to the pre-shock levels or below. While this response might be expected in intact animals, the same phenomenon was demonstrated in hypophysectomized dogs bled during constant infusion of ACTH.

In addition, it has been observed that infusion of oxytocin increased adrenal blood flow without changing corticosteroid secretion in both intact and hypophysectomized dogs, indicating lack of influence of change in the one parameter on that of the other (91).

Macchi and Hechter found no correlation between adrenal blood flow and corticosteroid output in perfused, isolated cow adrenals (103), and McDonald and Reich observed no influence of intravenous injection of ACTH on flow in transplanted sheep adrenals (111).

On the other hand, Walker and co-workers have emphasized the importance of adrenal blood flow on total 17-OHCS output in dogs (197). The influence of adrenal flow on corticosteroid secretion in rats has also been demonstrated by Porter and Klaiber (153). During constant infusion of ACTH into hypophysectomized rats in the former study, collection of blood and change in adrenal blood flow was performed by a "withdrawal" pump connected to the adrenal vein. With an increase in flow, i.e., greater withdrawal, adrenal corticoid secretion increased from 1.1 to 7.2 μg/5 min, with apparent maximal adrenal corticoid

output at a flow of 0.034 ml/min. While their very method of sampling and measurement of flow was obviously questionable, the authors suggested that a possible explanation for many discrepancies relating adrenal flow and corticoid secretion in rats has been failure to ligate the inferior phrenic vein and other tributary vessels emptying into the adrenal vein (153, 162). Without ligation, change in flow of adrenal effluent blood reportedly might not indicate a change of blood flow through the adrenal, but rather through the tributary vessels alone (153, 162). However, barring any change in secretion level, calculation of adrenal corticoid output would be the same in spite of dilution from tributary vessels.

Adrenal blood flow in rats was measured by Sapirstein and Goldman by a cardiac fractionation technique employing both Rb^{86} and iodoantipyrine (I^{131}) (162). With this method, adrenal blood flow was 0.14% of the cardiac output, which is equivalent to 0.078 ml/min or 1.9 ml/gm adrenal/min. This is somewhat lower than 5.05 ± 0.3 ml/gm adrenal/min measured in dogs (73). With the administration of large ACTH doses in rats, adrenal blood flow increased 114% and with the ligation of one common carotid artery the increase was 80% (162). However, surgical trauma had only minor effects on the estimated adrenal blood flow in those animals.

Further Rb^{86} studies indicated that while adrenal medullary perfusion per weight of tissue was greater than adrenal cortical

perfusion, increase of medullary blood flow and cortical flow in response to stress of unilateral carotid ligation were of the same order of magnitude. However, since the cortex comprises 85-95% of the total gland weight, the increase in flow to the cortex predominates (92). This evidence was interpreted to indicate that increase in flow to the cortex does not occur at the expense of diversion from the medullary vessels and does not support the concept of a cortico-medullary portal pathway (92).

In dealing with the adrenals of rats in a structural, cellular-vascular relationship, Lever concluded that three basic mechanisms control blood flow through the suprarenal glands: direct action of corticotropin on the blood vessels, nervous control of cortical arteries, and a slower control system through the degree of vasocompression of cells in the lucid zone (the zona intermedia and outer fascicular zone) (93). The cell size and lipid content of cells in the latter system appear to be related to anterior pituitary activity.

Recently, Maier and Staehelin suggested that ACTH may influence increased adrenal blood flow via the release of unsaturated fatty acids from cleavage of cholesterol esters during corticoidogenesis (106). The adrenals are particularly rich in tetraenoic acids, and these can be easily converted to prostaglandins. While a reduced form of these "essential" fatty acid products increases blood pressure in dogs, the

typical effect of prostaglandin compounds is vasodilation of the small peripheral arteries (9).

Intra-arterial infusion of ACTH, 1-60 mU ACTH/hr, in sheep with carotid-jugular adrenal transplants failed to produce an increase in blood flow, even though a considerable increase in cortisol and corticosterone and a slight rise in aldosterone output were recorded (203). When administered intravenously, however, doses between 1-25 U/hr elevated blood flow in all cases, even by 200%, and steroid outputs were raised upwards of 50% above that during the intra-arterial infusions. Beaven, Espiner and Hart recorded a significant elevation in adrenal blood flow in transplanted sheep adrenals with large doses of ACTH (1000 mU), less significant rises with injections of 20 and 50 mU, and no detectable difference with doses of 5 mU or less (6).

Following decerebration and hypophysectomy, adrenal blood flow in calves dropped from 10-20 ml/min to 5 ml/min by the end of the first hour (4). Intravenous infusion of small amounts of ACTH caused a temporary rise in adrenal flow in animals older than 10-40 days of age, but flow change was small or absent in animals less than 8 days old.

The rubidium method for measurement of adrenal blood flow with ACTH yielded the same results in dogs as those of Sapirstein in rats, i.e., increase in adrenal flow with large ACTH doses (178). As

measured by heated thermocouples, the same investigators recorded an increase in adrenal flow within one minute of ACTH administration in both anesthetized and conscious dogs.

After administration of a large ACTH dose, 5 units, to hypophysectomized dogs, Monos, Koltay and Kovach observed the "expected" elevation of corticoid output but only a slight increase in adrenal blood flow (122). The lack of a greater response was attributed to nearly complete dilation of the "ACTH-sensitive" vascular bed of the adrenal after hypophysectomy.

Within 2-5 minutes after injection of 25 units of ACTH into the peripheral circulation of dogs, Nicols and Richardson recorded a marked increase in adrenal blood flow which attained a maximum of 260-400% of control within 8-15 minutes (134). Pre-ACTH flow rates were restored after another 5-10 minutes, giving a total reaction time of 15-30 minutes.

The insecticide DDD, 2,2-bis(parachlorophenyl)-1,1-dichloroethane, is an isomer of DDT (dichlorodiphenyltrichloroethane), which along with another isomer, o,p' DDD, in low doses, produces reversible depression of adrenal 17-OHCS secretion and causes the adrenals to become refractory to ACTH stimulation (8, 128). With larger doses of DDD, necrosis and atrophy of the zona reticularis and fasciculata of the adrenal cortex were seen (8). Following ACTH administration to DDD treated

dogs, the "usual" increase in adrenal blood flow was observed, while steroid secretion remained low (134). These data were interpreted to indicate that the vasodilating factor of commercial ACTH is separate from the corticosteroid releasing factor, and the vasodilating effect may possibly be exerted via the medullary or extracapsular vasculature.

While the vasodilatory effects of the relatively impure clinical ACTH and the use of grossly pharmacologic doses were considered as possible explanations for the increased adrenal blood flow in DDD treated dogs, these possibilities were overruled (134). In a similar vein, Hartman, Brown and Liu have expressed the likelihood that increase in adrenal blood flow in dogs with injection of ACTH could be accounted for by contamination of the corticotropin preparation with posterior lobe pressor substances, these evoking an increase in pressure similar to that with Pitressin (73).

Because of the increase in adrenal flow and lack of corticosteroid elevation with ACTH in DDD treated dogs, Nichols and Richardson concluded that the vasopressor activity of commercial ACTH is separate from the corticoid forming and/or releasing factor (134). It was suggested that the site of vascular response of ACTH activity might reside in the vessels of the medulla or in the extracapsular vessels.

Holzbauer and Vogt similarly concluded that increase in adrenal flow caused by administration of ACTH in rats is not linked with its

synthetic action on the cortical tissue (86). This increase in flow occurred in spite of a decrease in femoral arterial pressure.

Adrenal blood flow through perfused dog adrenals has been demonstrated by Urquhart to be a significant parameter in the response of the glands to ACTH, and the steady state of steroid secretion by the adrenals is reportedly a reliable index of corticotropin release if the rate of adrenal flow does not vary (187, 188). The effect on flow was observed to be greatest at ACTH concentrations between 1-30 mU/min but was considerably reduced or absent at higher peptide concentrations or in the absence of ACTH. In addition, adrenal blood flow was approximately linear to perfusion pressure, i.e., arterial pressure in vivo (187, 188).

When the blood concentration of ACTH was held constant in the perfused adrenals, and the adrenal blood flow was approximately doubled, there occurred a 2-5 fold increase in the steady state secretion of corticoids (187). It was noted that at the concentrations of ACTH employed, an increment increase in flow rate at a fixed ACTH concentration always evoked a greater adrenal response than did a comparable increment produced by increasing the ACTH concentration at a fixed flow rate. Thus, the rate of presentation of corticotropin to the adrenal glands was better correlated with the steady state of corticoid output than was the blood concentration of ACTH.

The varied reports of the effect of ACTH on adrenal blood flow appear quite confused and conflicting. However, it will be noted that those cases reporting increase in flow with ACTH stimulation were effected by pharmacological doses. This is exemplified by Wright's findings of elevated flow with high ACTH infusion rates, but little effect at normal plasma ACTH levels and even at circulating ACTH levels found in patients with Addison's disease (194). The findings of the present study coincide with this concept in that hypophysectomy produced no change in blood flow that could be attributed to the rapid diminution of circulating ACTH.

Effect of Constant Infusion of ACTH In Hypophysectomized Dogs

Graded Rates of ACTH Infusion, Overshoot Response

The drop in ACTH level following hypophysectomy, as previously described, is indicated by the decline of 11-OHCS from a high of 6-9 μg/min/10 kg to a low of 0.1-0.2 μg/min/10 kg. This drop denotes a 30 to 90 fold decline between the surgical steroid output and the post-hypophysectomy low values. In order to arrive at a reasonable estimate of the adrenocorticotropic activity responsible for this range of steroid secretion, porcine ACTH was constantly infused in graded concentrations into hypophysectomized, adrenal-cannulated dogs.

Data on the effect of graded ACTH infusion were collected from 5 animals. Because of variabilities throughout the experiments, it was considered reasonable to discuss the data in terms of individual animals. The adrenal 11-OHCS output measurements at given rates of ACTH infusion showed some variation between individual animals. However, rates of ACTH infusion sufficient to produce minimal increases in 11-OHCS output from base levels and to evoke maximal adrenal stimulation were comparable in all cases.

Figure 14 shows a representative experiment illustrating the effect of graded ACTH infusion on 11-OHCS output. The pre-hypophysectomy samples gave a mean 11-OHCS output of 8.12 μg/min/10 kg, SD 0.37. In this particular experiment, adrenal blood was not sampled during the fall of ACTH immediately after hypophysectomy, and the three samples between 110 and 130 minutes indicated that the 11-OHCS level had declined to about 0.22 μg/min/10 kg.

At 137 minutes after hypophysectomy, ACTH infusion was begun at a concentration of 0.1 mU/ml, which at the pump speed of 0.17 ml/min produced an infusion rate of 0.017 mU/min. Within 2 to 3 minutes a sharp rise in 11-OHCS output was noted, and this reached a plateau of 0.56 μg/min/10 kg after about 30 minutes of infusion.

At 172 minutes post-hypophysectomy the infusion rate was switched to 0.051 mU/min, and within 2 minutes a sharp rise of 11-OHCS

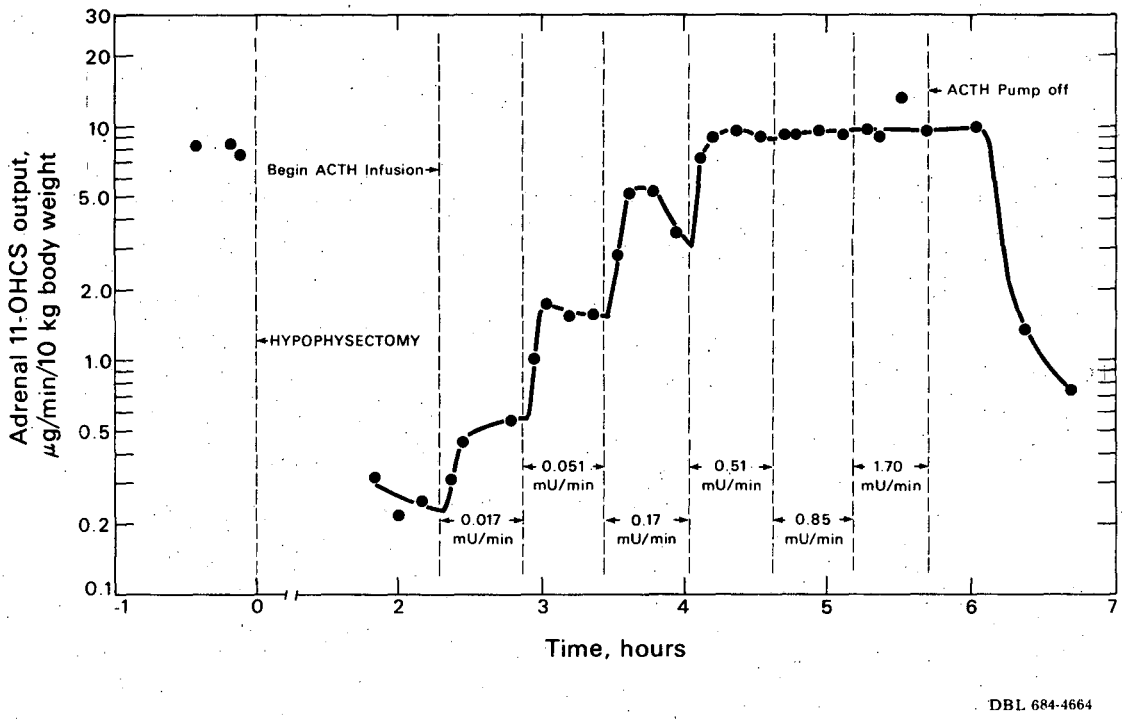

Figure 14. Adrenal 11-OHCS output during graded rates of ACTH infusion after hypophysectomy (Dog, D-209).

output was observed, this reaching a peak output of about 1.75 μg/min/10 kg within 10 minutes. After the next 24 minutes, the adrenal output appeared to stabilize at about 1.5-1.55 μg/min/10 kg.

The third ACTH infusion at 0.17 mU/min produced a pronounced "overshoot" following the rapid rise of 11-OHCS. A peak output of approximately 5.4 μg/min/10 kg was reached at about 16 minutes after the initial pulse of the new infusion.

A very dramatic elevation of 11-OHCS output was also observed with the infusion of corticotropin at 0.51 mU/min. The peak output of 9.6 μg/min/10 kg was reached in about 21 minutes, and an overshoot and decline in the 11-OHCS output, while considerably subdued, were suggested. Thirty-five minutes after the beginning of this infusion the output level was about 8.6 μg/min/10 kg.

With ACTH infusions at 0.85 mU/min (5 mU/ml) and 1.7 mU/min (10 mU/ml), no further elevation of corticoid output was observed from the previous peak of infusion at 0.51 mU/min (3 mU/ml). While a slightly rounded output profile can be imagined during the 0.85 mU/min infusion, adrenal output remained steady at about 9.4 μg/min/10 kg throughout the 0.85 mU/min and the 1.7 mU/min infusions.

The effect of constant infusion of graded concentrations of porcine ACTH into the venous circulation of hypophysectomized dogs is a stepwise increase in adrenocorticosteroid output. The levels of corticotropin

infused in this study indicated a range of ACTH activity for detectable changes in adrenal 11-OHCS output up to an ACTH infusion rate of about 0.85 mU/min. At that level, the adrenals appeared to be maximally stimulated such that increase of the infusion to 1.7 mU/min did not elicit further increase in adrenal steroid secretion. This is not to say, however, that other physiological functions influenced either directly or indirectly by ACTH secretion were also affected to the same degree, but only that the adrenal 11-OHCS secretory capacity was maximally stimulated.

It should be noted that the mean pre-hypophysectomy adrenal output of 8.12 μg/min/10 kg was only slightly different from the average of 9.4 μg/min/10 kg attained with the higher ACTH infusions of 0.85 and 1.7 mU/min. These data suggest that the adrenal glands, at least in this case, were functioning at near-maximal secretion from stimulation by endogenous corticotropin during the surgical, pre-hypophysectomy period. The high level of ACTH release during that period may have been responsible for the latent period before corticoid decline following hypophysectomy.

Several authors agree that the effect of operative trauma is sufficient to produce very high levels of adrenal stimulation. Administration of maximally stimulating doses of ACTH into both intact and hypophysectomized dogs has evoked secretion of adrenal corticosteroids

comparable with levels produced during surgery (78, 89). In studies on perfused adrenals, Hilton and co-workers suggested that blood obtained during surgery from non-hypophysectomized dogs contained sufficient ACTH to produce high and possibly maximal adrenal steroid secretion (84). On the other hand, it has been demonstrated in dogs that higher levels of 17-OHCS secretion could be produced with maximally stimulating doses of ACTH than those evoked by surgical effects of adrenal vein cannulation and craniotomy (201). In humans, the adrenals are reportedly not maximally stimulated during surgery, since the administration of large doses of ACTH has been observed to produce additional rises in peripheral corticosteroid levels (133, 161).

In this study a distinct overshoot in adrenal 11-OHCS output was observed with the infusion of ACTH at 0.17 mU/min, and a suggestion of the same phenomenon occurred with the infusion of ACTH at 0.051 and 0.51 mU/min. This overshoot response with step infusions of ACTH has been observed and discussed by Urquhart and Li (190). The overshoot was not apparent with the infusion of ACTH at 0.017 mU/min nor at 1.7 mU/min, and possibly did not actually occur at 0.85 mU/min. However, from the trend of the output values during the 0.85 mU/min infusion, a broad curve and possibly a suppressed overshoot can be imagined.

From these data it would appear that the overshoot phenomenon induced by a sudden increase of ACTH is suppressed at levels near both

minimal and maximal adrenal stimulation levels. Urquhart and Li also noted little or no overshoot relative to the steady state increment when the perfused glands were driven to near maximal rates of corticoid secretion at ACTH inputs of 30 to 70 μU/ml (190). It is suggested that in the physiological range of ACTH secretion, between 0.05 and 0.17 mU/min observed in this study, the influence of a sudden increase in ACTH on the adrenal cortex is to cause the release of corticosteroids held in reserve, or the rapid synthesis of corticoids from readily available precursors in the fascicular cells.

With the depletion of glucocortical "reserve" or of available precursors, a level of corticoid output would then be established which would be dependent on the level of stimulation by corticotropin and the availability of metabolites. At near minimal levels of adrenal stimulation, the influence of ACTH may be insufficient to cause a sudden release of corticoid "reserve" or the rapid conversion of precursors, but would be capable of a more smoothly graded, monotonic increase of steroid output. At maximal and near-maximal levels of corticoid secretion, the adrenal "reserve capacity" and precursor level may be at the point of near depletion, such that any sudden increase in ACTH stimulation would be without influence other than maintaining the adrenal output at that determinate maximal plane.

Twenty minutes after the termination of ACTH infusion at 1.7 mU/min in Figure 14, a high 11-OHCS output of 9.97 μg/min/10 kg was

still apparent. Shortly thereafter, the corticoid output began to fall and produced readings of 1.34 μg/min/10 kg at 40 minutes and 0.73 μg/min/10 kg at 60 minutes. In spite of the lack of a greater number of points during the output decline, a rough estimation gave a half-life for exogenous ACTH of about 4 minutes.

The prolonged shoulder following interruption of the last infusion was common in all cases in which the rate of ACTH infusion was in excess of that to produce maximal adrenal corticosteroid secretion. It must be remembered that the only index of ACTH activity in this study was measurement of adrenal venous corticosteroids. Thus, in cases in which the ACTH infusion rate was in excess of that sufficient to produce maximal corticoid secretion, the kinetics of ACTH activity were obscured. It is conceivable that prolongation of the latent period for corticoid decline following excessive ACTH influence might result from extended synthesis and/or release of corticosteroids as a consequence of a higher level of ACTH stimulation.

The overshoot phenomenon and step increase with graded ACTH infusion is also illustrated in the 11-OHCS output data shown in Figure 15. In this situation hypophysectomy was decidedly incomplete, but the extent of remaining hypophyseal tissue was not determined. The two pre-hypophysectomy adrenal plasma samples gave very widely separated output readings of 5.21 and 0.75 μg/min/10 kg at -14 and -5

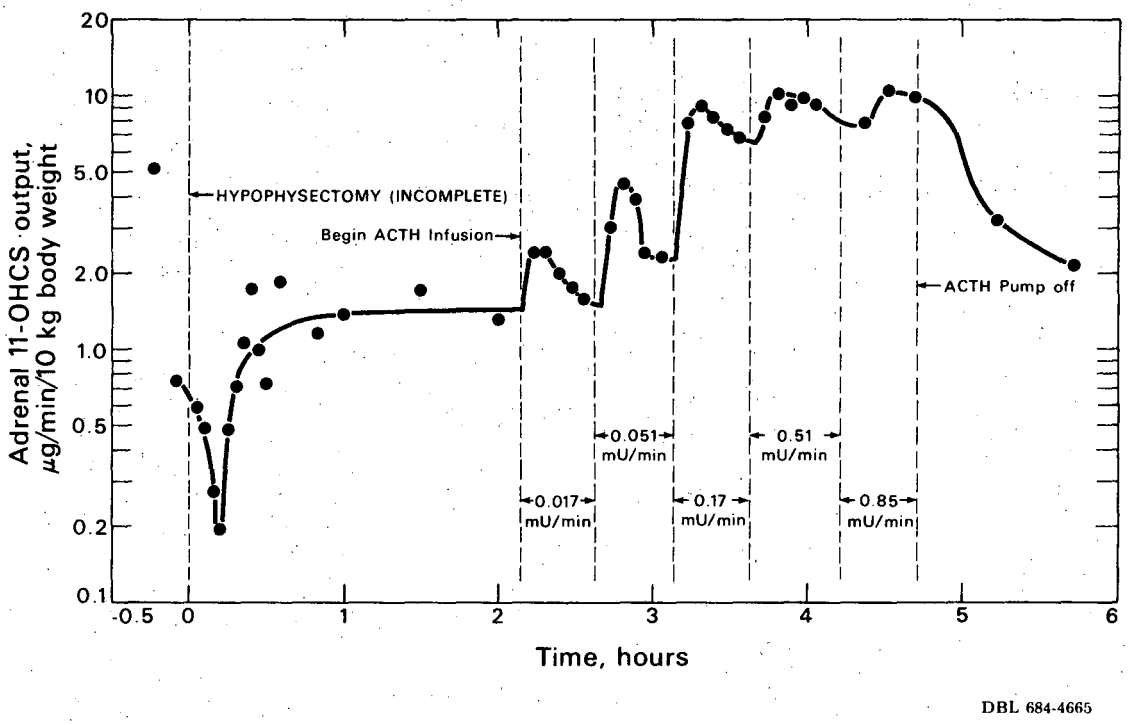

Figure 15. Adrenal 11-OHCS output during graded rates of ACTH infusion after incomplete hypophysectomy (Dog, D-29).

minutes respectively. At the time it was thought that the low level of the latter was the result of some technical error, but it soon became evident from the low trend of the following corticoid values that some pituitary damage had occurred before the estimated time of hypophysectomy, and that, indeed, hypophysectomy was incomplete. The lowest post-hypophysectomy level at 12 minutes was quickly followed by a sharp increase in 11-OHCS output reminiscent of that seen with the infusion of exogenous ACTH.

Following infusions of exogenous ACTH at 0.017 mU/min, 0.05 mU/min, 0.17 mU/min, 0.51 mU/min, and 0.85 mU/min, a sharp rise in steroid output was observed in each case as well as an overshoot and decline of the steroid level. The overshoot during the infusion of ACTH at 0.05 mU/min was somewhat faster than that seen at the other infusion rates and in the experiment presented in Figure 14. In that case, after the rapid rise of corticoid output with each infusion, the decline was more gradual and did not produce a steady level of secretion during the 30 minute periods of observation.

It is apparent that the peak output level attained with corticotropin infusion of 0.85 mU/min did not rise above that during the 0.51 mU/min infusion. These observations are in concert with those in Figure 14 with complete hypophysectomy, in which maximal adrenal stimulation occurred at rates of ACTH infusion between 0.51 mU/min and 0.85 mU/min.

Only two adrenal samples were obtained after the termination of corticotropin infusion. In spite of the lack of a sufficient number of points to establish a concise decay curve, it is apparent that endogenous corticotropin from hypophyseal remnants was sufficient to maintain the 11-OHCS output level at a relatively high plane.

It was considered that subtraction of the estimated endogenous ACTH level, i.e., the resultant adrenal steroid output after partial hypophysectomy, from the corticoid output during the graded exogenous infusions might provide useful information comparative to data from complete hypophysectomy. After subtraction, the peak 11-OHCS outputs produced by ACTH infusions of 0.017, 0.05 and 0.17 mU/ml were approximately 0.5, 1.5 and 2 μg/min/10 kg, higher than the respective peak outputs at comparable infusion rates in the hypophysectomized dog. By subtracting the endogenous component, the sharpest overshoot would then be produced at the lowest infusion rate of 0.017 mU/ml. If the premise presented above is assumed to be correct, that an overshoot is not produced at low rates of ACTH infusion, though a monotonic increase in corticoid output may be produced, then the existence of a sharp overshoot at such a low rate of infusion is inconsistent with data from hypophysectomized dogs. It is suggested from these observations that the dynamics of ACTH activity at low and possibly at high levels, as determined by 11-OHCS output, may not be generalized with respect

to activity nearer the mid-physiological range, but must involve additional considerations.

Evidence from the preceding experiments indicates that ACTH infused into hypophysectomized dogs at a dose as low as 0.017 mU/min (0.1 mU/ml) is sufficient to produce a perceptible elevation in corticoid output and is even sufficient to elevate the corticoid output in partially hypophysectomized dogs in which the output is as high as 1.4 μg/min/10 kg. If an analogy can be drawn between the infusion rate of exogenous ACTH, based on corticoid output in hypophysectomized dogs and the rate of secretion of endogenous corticotropin in partially hypophysectomized dogs, it may be estimated that the ACTH secretion rate sufficient to maintain a corticoid output of 1.4 μg/min/10 kg is in the range of 0.051 mU/min with a blood concentration of 0.3 mU/ml. Maximal adrenal stimulation appears to occur with an exogenous ACTH infusion rate of 0.85 mU/min and an estimated blood ACTH concentration of 5 mU/ml, this producing an adrenal corticoid output in the range of 10 μg/min/10 kg.

While complications were encountered at lower infusion rates of ACTH illustrated in Figure 16, near-maximal adrenal 11-OHCS output was produced with an infusion rate of 0.17 mU/min, and only a slightly greater output was elicited with an ACTH infusion rate of 17 mU/min. It should also be noted that with the termination of ACTH infusion at 17 mU/min, the 11-OHCS output remained high and did not begin to fall for

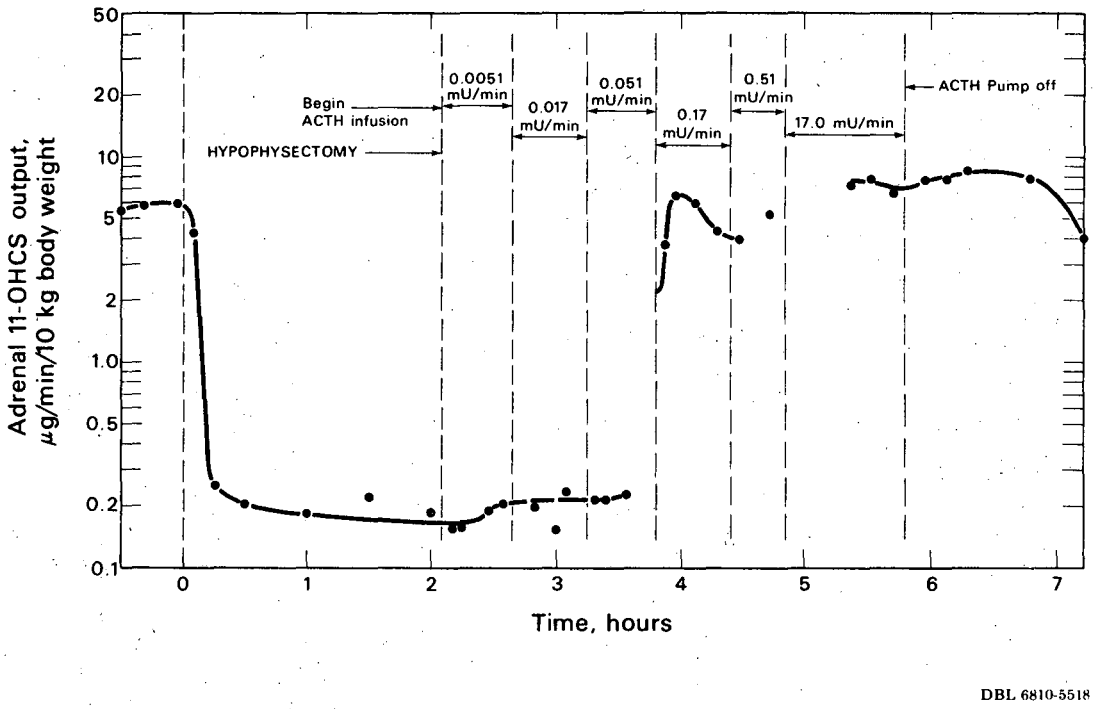

Figure 16. Adrenal 11-OHCS output during graded rates of ACTH infusion after hypophysectomy (Dog, D-198).

about 45 minutes. Even by 80 minutes, the usual drop seen after hypophysectomy had not occurred.

A very rapid response of the adrenals to a pulse stimulation of exogenous ACTH has been observed in a number of species. In rats an increase in corticosterone secretion has been recorded at 4 minutes (101) and 2 minutes (102) after systemic injection of ACTH. The adrenal response occurred "momentarily earlier" than 2 minutes with administration of ACTH in a retrograde fashion (102). A note of interest in the former study was that ascorbic acid released into adrenal effluent blood (not to be confused with the adrenal ascorbic acid depletion method of ACTH assay) preceded that of corticoid release. As the steroid level rose a decline of ascorbic acid was observed, and in the absence of measurable corticoids no release of ascorbic acid was recorded. The temporal relationship and consistency of events suggest the need of further information of the ill-defined role which ascorbic acid may play in the synthesis and/or release of adrenal corticosteroid in response to ACTH (101).

In sheep with transplanted adrenals, large single doses of ACTH - 20, 50, and 100 mU - evoked rapid, maximal steroid secretion rates of 10-14 μg/min by the third minute after injection (6). In hypophysectomized, adrenal-cannulated dogs, a waiting period of 3 minutes was considered satisfactory for adrenal response to intravenously administered

doses of 1, 2, 5 and 10 mU of ACTH (130). With in situ perfusion of the adrenal gland of hypophysectomized dogs, step input of ACTH evoked an increase in corticoid secretion between 2-3 minutes (190, 191), and after single injections of ACTH, the cortisol output returned approximately to its original level within 30 minutes.

Perfusion of bovine adrenals with an ACTH concentration of 0.001 I.U. per liter (0.017 mU/min at a perfusion rate of one liter per hour) produced a definite increase in corticosteroid output, and a maximal steroid output was achieved with a concentration of 0.1 I.U. per liter (1.7 mU/min) (104). Even though samples were only obtained at 18 minute intervals, Macchi and Hechter asserted that maximal adrenal stimulation did not occur during the first 18 minutes, but maximal corticoid output did occur during the perfusion intervals of 18-36 minutes or 36-54 minutes. During the 54-72 minute intervals, corticosteroid output declined somewhat (104).

As emphasized by Urquhart and Li, the most strikingly unexpected feature revealed in their studies of adrenocortical dynamics was the overshoot response to a stepwise increase in ACTH perfusion (190). After the secretory response reached its peak, within about 11 minutes, the cortisol level declined slowly and did not reach a steady state of output until about 30 minutes. In addition, it was demonstrated that the overshoot response could be evoked in glands already stimulated by ACTH.

Inasmuch as the 11-OHCS overshoot responses were not followed for more than 30-31 minutes in this study, it was difficult to contrast time relationships with respect to output declination with those observed by Urquhart and Li (190). However, with the institution of greater inputs of ACTH, one peak occurred at an earlier time of 8 minutes (Fig. 15, 0.017 mU/min), several of the outputs peaked at 10 to 11 minutes - the time described by Urquhart (Fig. 14, 0.051 mU/min; Fig. 15, 0.051, 0.17, and 0.51 mU/min; Fig. 16, 0.17 mU/min), and two peaks occurred at 13-15 minutes (Fig. 14, 0.17 and 0.51 mU/min).

Only two overshoot responses showed the establishment of a corticoid output plateau within 30 minutes as described by Urquhart and Li (Fig. 14, 0.051 mU/ml and Fig. 15, 0.051 mU/ml) (190). The peak decline in the latter case occurred more rapidly with a steady 11-OHCS secretion by 22-25 minutes. In the other cases, the declination angle of the 11-OHCS output with the institution of succeeding ACTH infusions was such as to suggest a stable level of secretion at periods longer than 30 minutes.

Although sufficient evidence is lacking, where the overshoot response did occur, it appeared that peaking in shorter periods of time was elicited by lower doses of ACTH infusion, while the peaks at longer durations occurred in response to higher levels of trophic stimulation. Urquhart and Li, in two separate glands, noted a longer time factor in

the higher corticoid peak responses with a single injection of 2,500 microunits[1] of ACTH than that following injection of 250 microunits (190). In addition, two overshoot responses were produced by two identical step infusion changes from 0 to 2 μU/ml, 40 minutes separating the infusion changes, while no overshoot was evoked in the second change when the interval between the infusions was only 5 minutes (190). The overshoot phenomenon of adrenocorticosteroid secretion in response to increase of ACTH stimulation appears to be dependent both on the extent of change of ACTH input and on time (190).

While not specifically expressed as such, an overshoot response of adrenal corticosterone output to ACTH infusion in rats was suggested from the data of Porter and Klaiber (152). Even though ACTH infusion was begun within 30 seconds after hypophysectomy, and adrenal blood samples were collected over 15 minute periods, infusions of 26 μU/min and 52 μU/min produced elevated levels of corticosterone during the first 15 minutes and somewhat higher values during the second 15 minutes. Lower steroid values were recorded between 30 and 45 minutes and stable levels of corticoid secretion were attained between 45 and 60 minutes.

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[1] The shift in reference from mU to μU dilutions of ACTH resulted from infusions in vivo versus perfusion of isolated adrenals respectively.

Undershoot Response

A feature observed in some cases of incomplete hypophysectomy, although usually not as accentuated as that in Figure 15 (Fig. 17 example A), was an "undershoot" of adrenal 11-OHCS output.

A cortisol undershoot response to stepwise decrease in ACTH concentration has been described by Urquhart and Li in perfused dog adrenals (190). They expressed the phenomenon as a "slight tendency to undershoot the steady-state rate of cortisol secretion" to a degree considerably less than the overshoot produced by an increase in ACTH input.

The undershoot described in Figure 17, example A, is quite profound and has been seen in other cases of incomplete hypophysectomy. The usual situation, however, is a more subdued undershoot as shown in Figure 17, examples B and C, with a less dramatic rise to the steady-state output. In some cases of incomplete hypophysectomy the undershoot was absent, e.g., Fig. 17 example D.

It can be imagined that the drop of influence of endogenous ACTH activity on adrenal corticoid output with incomplete hypophysectomy might be similar to a sudden drop in the rate of exogenous ACTH infusion after hypophysectomy: the new level of ACTH activity in the former case would be dependent on the extent of remaining pituitary tissue. However, further speculation comparing the responses shown in Figure

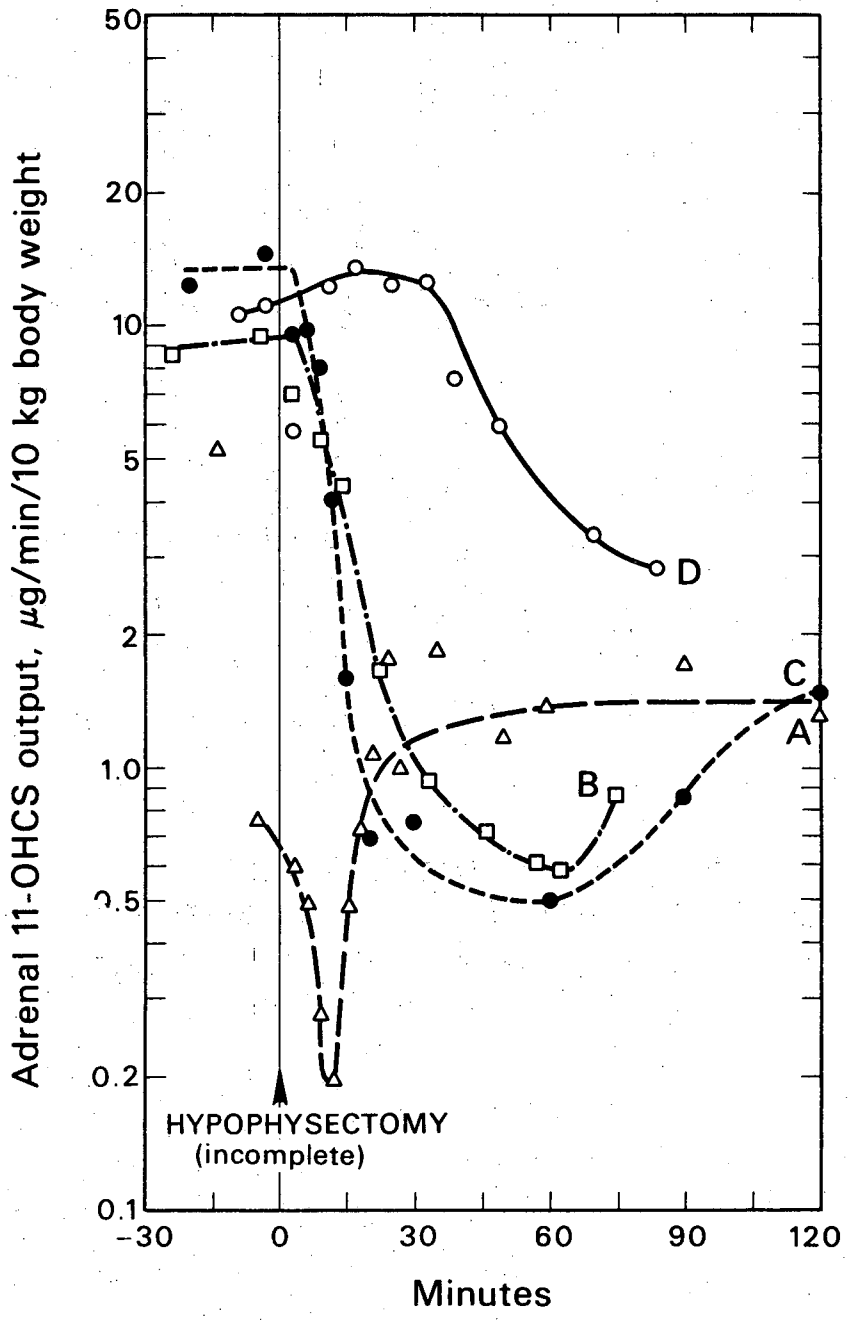

Figure 17. Examples of adrenal 11-OHCS output following incomplete hypophysectomy.

17 with the cortisol undershoot of Urquhart and Li (190) would be fortuitous in the absence of more conclusive data.

Mechanism of ACTH Stimulation of the Adrenal Cortex

The step, or steps, by which ACTH accelerates steroidogenesis have as yet been only imprecisely determined. Several studies indicate that the influence of ACTH is in a single rate-limiting step between cholesterol and pregnenolone and not later than the hydroxylation of C-17, C-21 and C-11β. According to this evidence, the corticotropic influence is believed to act in the conversion of cholesterol to 20 α-hydroxycholesterol (77).

On the basis of increased intracellular corticosteroid content of bovine adrenals, Hayes and Berthet concluded that ACTH stimulates corticoid synthesis rather than release (75). From this work it was suggested that the effect of ACTH is to increase phosphorylase activity and to convert glycogen to glucose-1-phosphate. Glucose-1-phosphate is then rapidly converted to glucose-6-phosphate, which is metabolized by dehydrogenation. In the process, reduced TPN is generated, which then stimulates the processes of corticoid synthesis.

In a later study, Hayes, Koritz and Peron observed that adenosine 3', 5'-monophosphate, when added to rat adrenal slices in vitro, elicited an increase in steroid synthesis comparable to, if not greater than, that

with the addition of ACTH (76). Addition of substances closely related to 3', 5'-AMP had no effect.

Hilton et al., in finding that perfusion of 3', 5'-AMP into intact dog adrenals caused continued production of corticosteroids in the absence of ACTH, reinforced Haynes, Koritz and Peron's theory of an ACTH-phosphorylase related mechanism (75, 76, 83). The same authors (83) noted a species difference in the sensitivity of adrenal tissue to both 3', 5'-AMP and ACTH. Rat adrenals, which responded with similar corticosterone outputs to comparable increments of 3', 5'-AMP and ACTH, exhibited a greater sensitivity than did the adrenals of guinea pigs. Perfused dog adrenals appeared to be the most sensitive. It should be pointed out that Macchi and Hechter observed a greater corticoid output in lacerated venous-cannulated bovine adrenals than in arterially perfused preparations, and emphasized that, both in the presence and absence of ACTH, corticosteroid output was markedly influenced by the method of preparation of the adrenal gland (104).

In addition to considerable evidence for the effect of ACTH acting via cyclic 3', 5'-AMP in the release of adrenal corticosteroids, it is generally agreed that an increase in adrenal phosphorylase activity follows the influence of corticotropin (82). According to the Haynes and Berthet theory of the mechanism of ACTH activity (75), ACTH acts to stimulate the formation of cyclic AMP which in turn converts

phosphorylase from an inactive state into the active enzyme. After phosphorylation of glycogen to glucose-1-phosphate and the conversion to glucose-6-phosphate, metabolism of the latter results in the production of reduced TPN (TPNH).

The production of TPNH supplies the necessary cofactor for energy required in steroidogenesis. Further steps in the chain of events remain speculative, but a schematic mechanism derived from existing data has been proposed by Hilf in Figure 18 (82). In Hilf's review, nicotinamide adenine dinucleotide phosphate is referred to as NADP rather than TPN and its reduced form is referred to as NADPH rather than TPNH. Further detailed discussion of the mechanism of ACTH stimulation of corticosteroid secretion is found in reviews by Hechter and Halkerstom (77), by Hilf (82), by Yates and Urquhart (208), and in papers in McKerns (112, vol. 1).

In conjunction with the existing data implicating ACTH and 3', 5'-AMP in secretion of adrenocorticoids, Urquhart and Li have demonstrated that the elevation in cortisol secretion of the perfused dog adrenal was comparable with that of a stepwise increase of cyclic AMP in the perfusate (190). In addition, the extent of overshoot and the time course of the cortisol response to increases in 3', 5'-AMP did not differ from that seen with stepwise infusion of ACTH. In light of prevailing information, Urquhart and Li have proposed two model systems relating

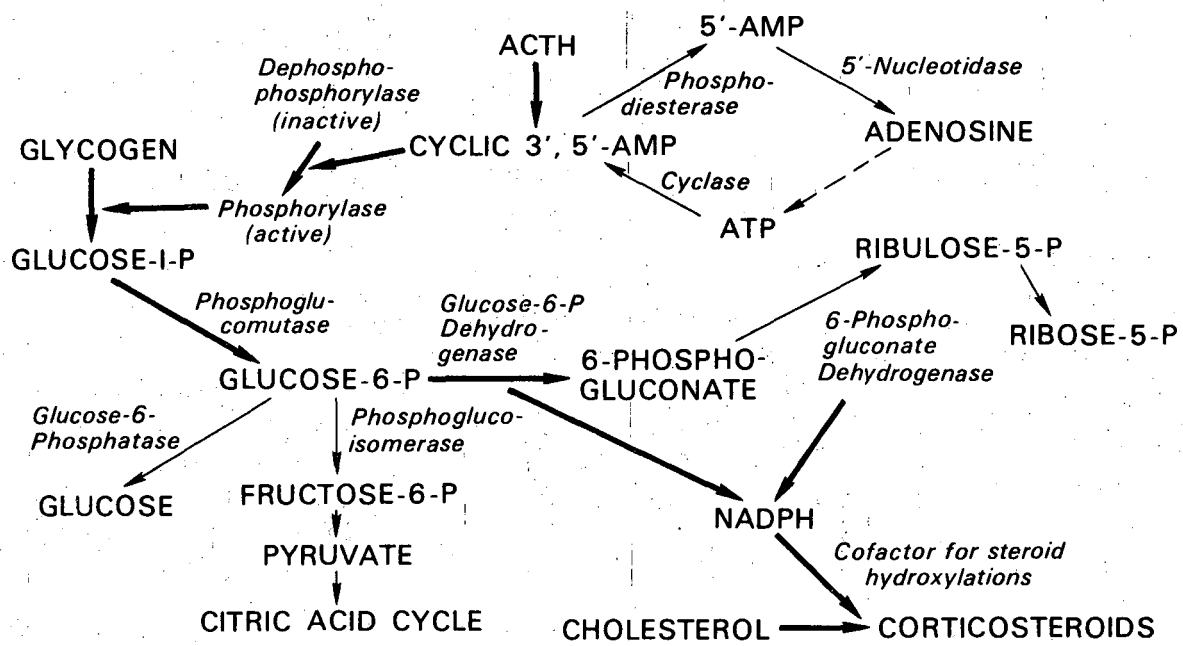

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Figure 18. Metabolic pathway associated with the action of ACTH on the adrenal cortex (Hilf, R., New Eng. J. Med. 273:798, 1965). Heavy arrows designate pathway proposed by the Haynes-Berthet theory. Lighter arrows indicate pathways associated with metabolism of intermediates formed as the result of ACTH stimulation of the adrenal glands.

ACTH activity to steroidogenesis (190). In spite of the possibilities that complexities of the overshoot phenomenon might ultimately reside in the interaction of cyclic AMP and cholesterol hydroxylation, these authors have concentrated the thesis of their models on the presumption that the rate parameter for cholesterol hydroxylation does not overshoot, but rather rises monotonically with increase in ACTH concentration. They, therefore, chose to postulate additional mechanisms in the explanation of the steroidogenic pathway. The equations and computations in the production of these model systems are explained in greater detail by Urquhart and Li (190).

In relating ACTH activity with adrenal secretion of corticosteroids, it should be considered that variability between data derived from different animals may reside at a number of points between the initial influence of ACTH and the secretion of adrenal steroids. Since the intermediates in the steroidogenic scheme, including enzymes, cyclic 3', 5'-AMP, and NADPH, are themselves subject to metabolic influences, it is reasonable that deviations in activity functions along the sequence of events would elicit somewhat different steroid output readings from individual animals.

11-OHCS Output - ACTH Dose Relationship

Within a restricted range, the output of adrenal corticoids has been reported to increase in a linear fashion as a function of the logarithm

of the ACTH dose. In normal humans receiving 24 hour ACTH infusions, Ney and co-workers found a rectilinear relationship between both plasma and urinary 17-OHCS and the logarithm of the plasma ACTH concentration (133). This relationship held as long as plasma corticotropin concentration did not exceed 3 mU/100 ml, above which the adrenals appeared to be maximally stimulated. These authors estimated a mean range of plasma ACTH concentration of between 0.25 mU/ml and 0.11 mU/100 ml during the normal circadian cycle. Single injections of ACTH in humans produced a rise of peripheral blood 17-OHCS level which reached a peak in approximately one hour and returned to the pre-injection level in 3 hours (132).

In rats, following single injections of corticotropic hormone, the level of corticosterone began to rise as early as 2 minutes after injection and reached a maximum in 5 - 8 minutes (102). With jugular injection of ACTH, a linear elevation of Compound B was obtained with log doses of ACTH between 0.05 and 1.0 mU, while a greater sensitivity, although less precise, was obtained with retrograde adrenal vein injections of log ACTH doses between 0.005 and 0.5 mU (102). Guillemin et al. determined a linear response of 17-OHCS secretion in rats to log ACTH doses between 0.2 and 1.0 mU (67). With constant infusion of corticotropin in rats, Porter and Klaiber observed that corticosterone increased linearly as a function of the log dose of ACTH with rates of

52, 78, and 104 μU/min (152). In that study it was estimated that ACTH secretion in rats subjected to experimental stress was between 26 and 52 μU/min.

During constant ACTH perfusion of bovine adrenals, Macchi and Hechter observed a linear response of corticosteroid output with log doses of ACTH between 0.001 and 0.1 I.U. per liter at a rate of one liter per hour (104).

The relationship between linearity of adrenal steroid secretion and the log dose of ACTH in dogs formed the basis for the bioassay technique for ACTH proposed by Nelson and Hume (130). In that study, involving 10 minute adrenal blood samples after single injections of ACTH, a linear corticoid response was noted with log ACTH doses between 1 and 10 milliunits. In perfused dog adrenals, Urquhart, Li and Montgomery observed an "approximate" linearity of response with log concentrations of ACTH in the range between 0 and 3 μU/ml (191).

In the present study the determination of a linear 11-OHCS-log ACTH relationship was complicated by the fact that in many cases observations were not extended to the point of definite steady states of 11-OHCS output with increasing ACTH levels. In those cases, it was possible to estimate a stable corticoid output after the overshoot, and those values along with the measured stable outputs were plotted against the log ACTH inputs in Figure 19A. From these data there is an apparent

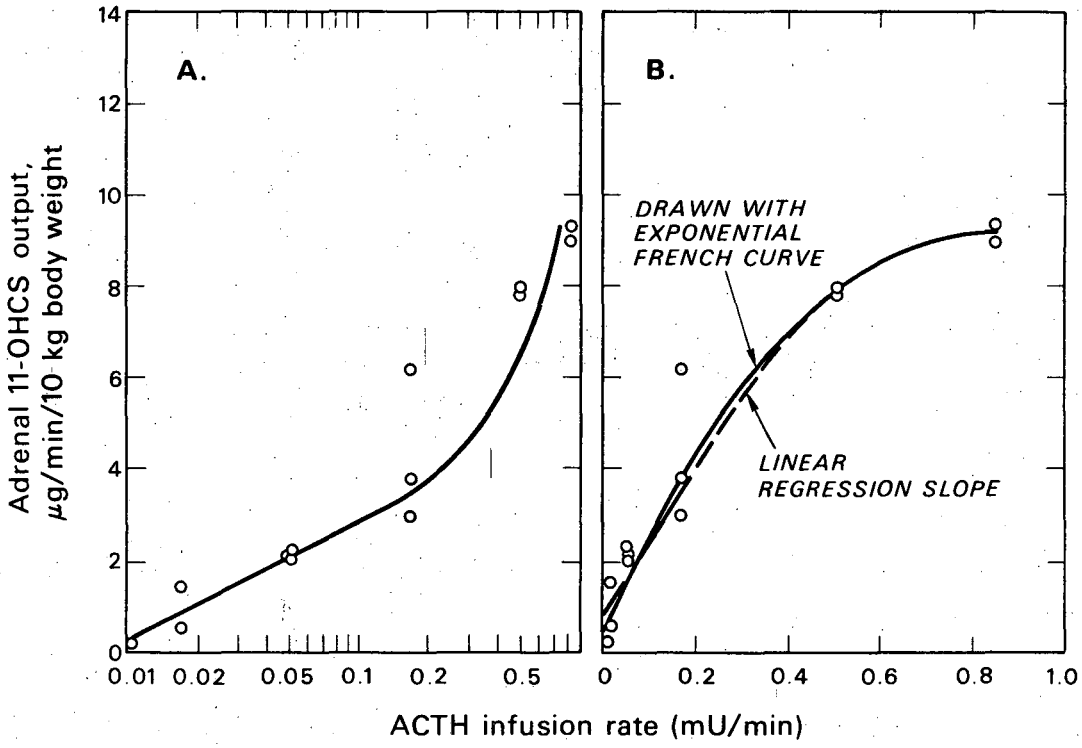

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Figure 19. Relationship between adrenal 11-OHCS output and rate of ACTH infusion. A. 11-OHCS output versus log ACTH dose. B. 11-OHCS output versus ACTH dose.

linearity in the increase in corticoid output with step increases in the rate of corticotropin infusion between 0.01 mU/min and 0.2 mU/min. Although no output values were measured for ACTH infusions between 0.17 and 0.51 mU/min, it was estimated from the trend of the points in Figure 19A that an infusion of ACTH at 0.2 mU/min forms the near-upper limit of ACTH input for the production of a linear steroid output.

11-OHCS output was also plotted against infusion rate on a linear scale as shown in Figure 19B. Because of the small number and proximity of the points in the lower range, it might be tempting to suggest a direct relationship between corticoid output and ACTH infusion rate in that range. However, it will be noted that a line drawn with an exponential French curve in Figure 19B, from estimation by eye, fits the trend of the points equally well. Thus, within a range of ACTH infusion between 0.01 and 0.5 mU/min, a linear corticoid response may be interpreted with either the ACTH dose or the logarithm of the ACTH dose.

As pointed out above, other investigators have suggested that adrenal steroid output increases in a linear fashion with the logarithm of the ACTH dose. Upon calculation of the circulating ACTH concentration the relationship

$$Q_c = K \log [ACTH]$$

is derived where Q_c is the adrenal corticoid output, [ACTH] is the ACTH concentration and K is a proportionality constant. Yet, in the determination of ACTH half-life based on the decline of adrenal corticoid output after hypophysectomy, it has been assumed in this and other studies that an exponential steroid depression reflects a proportional drop in the circulating ACTH level. With these considerations in mind, the existence of a linear log ACTH:corticoid output relationship might indicate a faster ACTH half-life than the 3.5 minutes determined previously.

Figure 19C illustrates the estimated circulating ACTH concentration as a function of time after hypophysectomy. This conversion was accomplished by correlating the 11-OHCS output after hypophysectomy, described in Figure 11B, with corticoid output produced by graded rates of ACTH infusion, see Figures 19A and 19B. Thus, ACTH values were derived assuming both linear ACTH:corticoid output and log ACTH:corticoid relationships.

It is apparent from Figure 19C that neither of the above assumptions accounts for an exponential disappearance of ACTH over an extended decline of the hormone. Assuming a linear ACTH:11-OHCS relationship, an exponential decline of ACTH appeared between circulating concentration levels of about 0.7 and 3 μU/ml, approximately a four fold drop. The $T_{1/2}$ for ACTH from this decline was 2.6 minutes

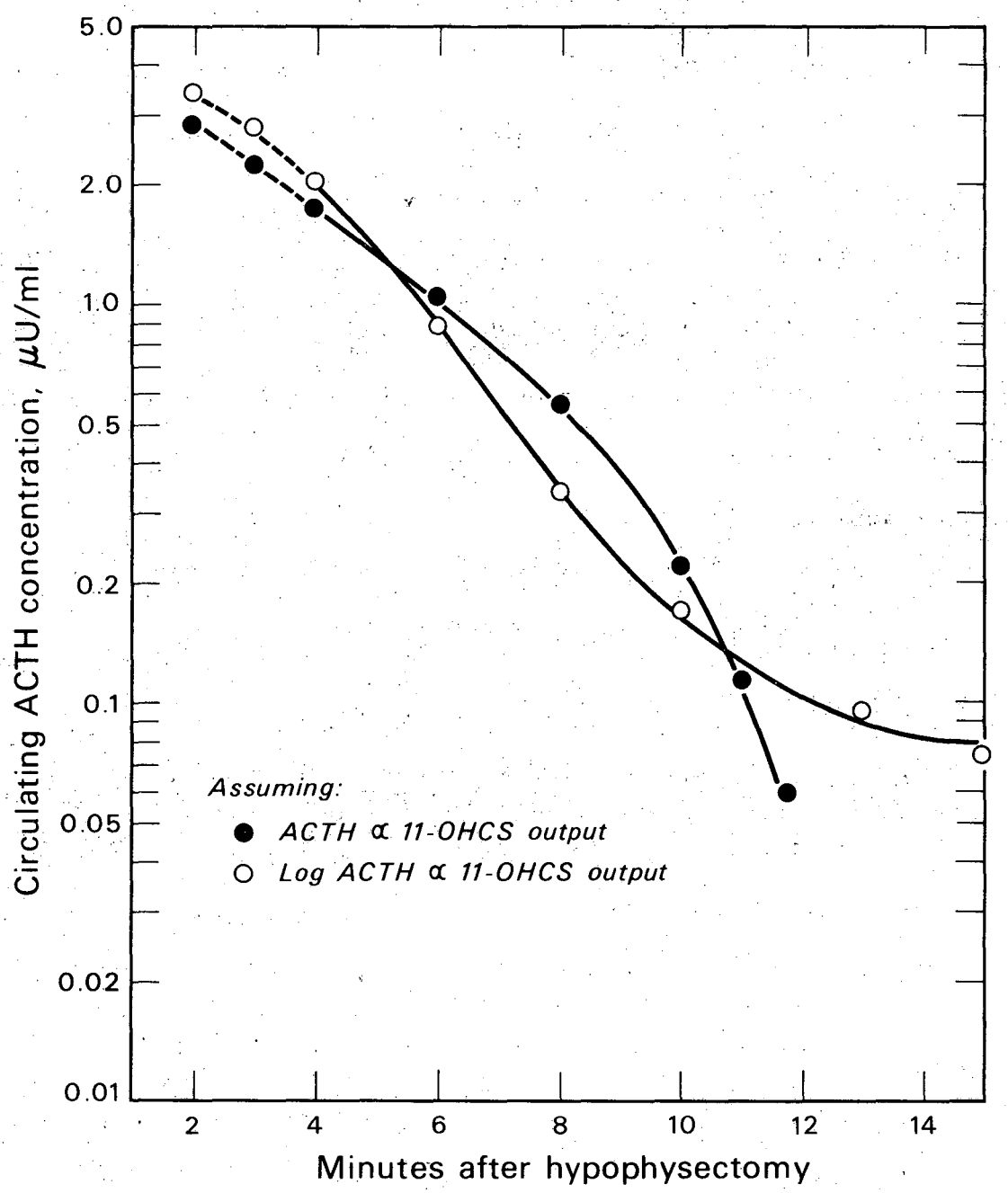

Figure 19C. Estimation of circulating ACTH concentration levels after hypophysectomy assuming linearity of both ACTH:11-OHCS output and log ACTH:11-OHCS output relationships.

which was within the 95% confidence limit range of 2.5-5.5 minutes determined previously.

Assuming a log ACTH:11-OHCS relationship, a sigmoid ACTH disappearance curve was produced, which exhibited an exponential trend between concentration levels of about 0.35 and 1.5 μU/ml, again about a four fold decline. The half-life for ACTH over this range, assuming an exponential decline, was 1.4 minutes, which was considerably faster than the $T_{1/2}$ of 3.5 minutes determined from the depression of adrenal 11-OHCS output after hypophysectomy. It was also well outside of the 95% confidence limit range.

It is apparent that the data presented here can neither defend the purported log ACTH:corticoid hypothesis nor establish a firm contention for a linear ACTH:corticoid relationship. It is possible that the explanation may lie somewhere between the two considered possibilities, or other factors may be influential. It is suggested, however, that there is evidence for a need for further evaluation of the interrelationship between the level of ACTH stimulation and adrenal corticoid output, the dynamics of which may vary over a wide range.

Interruption of Exogenous ACTH Infusion

If the effect of exogenous ACTH is the same as that of endogenous ACTH on the stimulation and release of corticosteroids from the adrenals, it would be expected that the fall of ACTH with interruption of infusion, as

reflected by the decline of corticoid output, would be similar to that produced after hypophysectomy. This does appear to be the case as shown in Figure 20. The time interval between interruption of infusion and the appearance of 11-OHCS decline, however, appeared to be longer at higher rates of ACTH infusion.

Each set of data in Figure 20 was derived independently from separate animals, and discrepancies in peak adrenal 11-OHCS output with maximally stimulating doses of ACTH were probably due to the individual sensitivity of the animals.

Following the lowest rate of ACTH infusion, 0.05 mU/min, 11-OHCS output began to decline after about 6 minutes. In spite of a small number of points for a well defined corticoid decline, an ACTH half-life of about 3 minutes was estimated following interruption of this infusion.

The infusion of ACTH at the estimated rate of 0.5 to 0.6 mU/min was performed with gelatin-free corticotropin described in the methods section. When this source of ACTH was obtained, the stated activity level was 30 U/mg and dilutions throughout several experiments were made on that basis. However, after detecting adrenal outputs incompatible with those produced by estimated comparable infusions of commercial ACTH, it was learned that the gelatin-free ACTH had actually not been assayed. An infusion rate of 2 mU/min calculated for the gelatin-free peptide was estimated to be equivalent to approximately

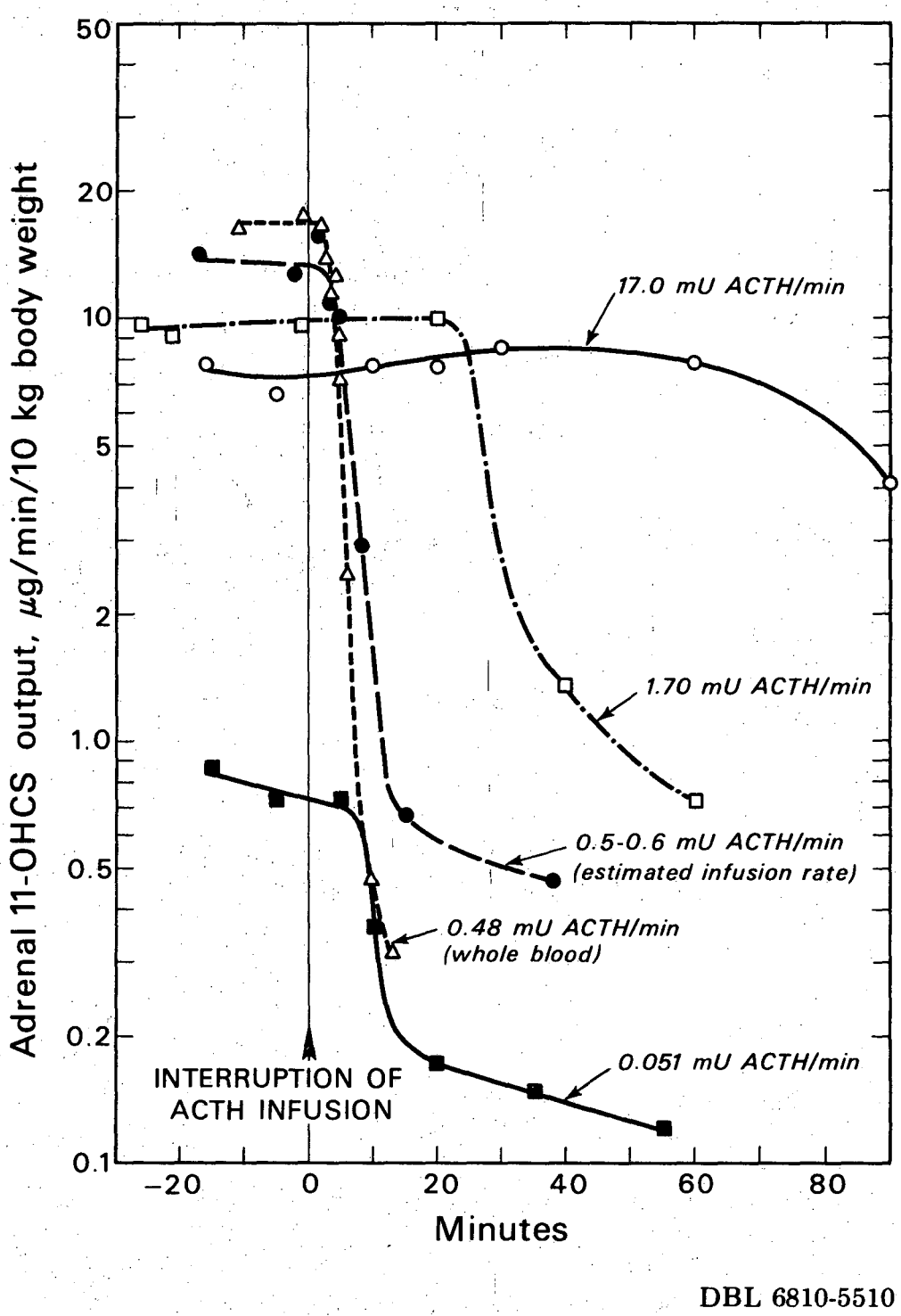

Figure 20. Adrenal 11-OHCS output after interruption of ACTH infusion in hypophysectomized dogs.

0.5 - 0.6 mU/min with the Armour Corticotropin. This is a level determined sufficient to produce submaximal stimulation of corticosteroid secretion.

With infusion of ACTH at 0.5-0.6 mU/ml an 11-OHCS output of about 13.5 μg/min/10 kg was attained, and within 3 to 4 minutes after cessation of infusion, the corticoid output dropped precipitously. From the relatively large number of steroid output measurements obtained following this infusion, it was possible to determine a half-life for ACTH of 3 minutes.

A great similarity in the decline of 11-OHCS was observed between interruption of infusion of ACTH in the previous example and that following infusion at 0.48 mU/min. Although different sources of corticotropin were employed in the two cases, the ACTH activity of the two was estimated to be essentially equivalent. In addition, with the infusion at 0.48 mU/min, 11-OHCS output was determined in whole blood, thus showing the similarity of results from measurement of corticoids in plasma and blood. The higher output of about 17 μg/min/10 kg attained during the latter infusion, however, was probably a reflection of this method of measurement.

Within 3 to 4 minutes after cessation of ACTH infusion at 0.48 mU/min, the 11-OHCS output level fell dramatically. The estimated $T_{1/2}$ for ACTH as indicated by 11-OHCS measurement in whole blood was about 1.5 minutes, suggesting an extremely rapid inactivation.

With the infusion of ACTH at 1.7 mU/min, a substantially maximal stimulating dose, a stable corticoid output of about 9.7 μg/min/10 kg was attained, but with interruption of infusion, the same output was maintained, and a decline was not detected for over 20 minutes.

With interruption of ACTH input at 17.0 mU/min, or a dose approximately 20 times greater than that necessary to produce maximal adrenal stimulation, an extremely prolonged corticoid output was maintained. Between 60 and 90 minutes a slow decline became apparent, but the 11-OHCS output fell to only 4.0 μg/min/10 kg by 90 minutes.

Ganong noted that while the corticoid response was not elevated above the maximal output, increasing single doses of intravenously injected ACTH in hypophysectomized dogs provoked a more prolonged output at higher ACTH dose levels (56).

Urquhart and Li reported an exponential decline in cortisol output within 2 minutes of stepwise decrease of submaximal ACTH in perfused dog adrenals (190). The half-life for exogenous ACTH was estimated to be approximately 2.5 minutes. An exponential decline of corticoid output was also recorded with termination of ACTH infusion in lacerated cow adrenal. However, in that case the $T_{1/2}$ was estimated at 25 minutes (105). In the latter experiment the stimulating dose of ACTH was about 10 times that considered sufficient to produce maximal adrenal corticoid output.

Prolonged adrenal response to ACTH has been observed in situ in dog adrenals "autoperfused" with blood from hypophysectomized donors (84). With a supramaximal stimulating dose of 25 U of ACTH over a period of 10 minutes, the hydrocortisone level rose immediately and remained at the elevated level for "at least 2 hours." In transplanted sheep adrenals extended responses of steroid output were also reported with maximal stimulating doses of ACTH - 20, 50 and 1000 mU (6). The extended times before fall in corticoid output appeared to be associated with higher doses of ACTH.

As pointed out by Urquhart and Li (190), prolongation of elevated corticosteroid outputs, observed in the studies with interruption of ACTH infusion at high stimulating doses, may bear on reports of ACTH binding by adrenal cortical cells. The same may be true in the present study. Birmingham and Kurlents demonstrated a slow decline in secretion of corticosteroids from rat adrenal slices with only brief exposure to ACTH (10). Those investigators suggested a binding of the peptide and prolonged stimulation of the target tissue comparable with that seen in the binding of insulin by muscle. The findings of Richards and Sayers lent some support to this postulate in noting an appreciable content of ACTH activity in adrenal tissue after injection of corticotropin into intact rats (157).

There appears to be some difference in adrenal corticoid response between cases of short single pulse stimuli of ACTH to the adrenals, of

step decrease and total interruption of high ACTH doses, and between instances of step decrease and total interruption of submaximal corticotropin stimulation. Sharp declines in cortisol output were recorded with stepwise decreases of ACTH in perfused dog adrenals, whereas with single injection of corticotropin, even submaximal, cortisol output was more prolonged with a slower decline (190). In the present study, hypophysectomy and interruption of ACTH infusion up to maximal stimulating doses of ACTH produced a rapid fall in 11-OHCS output, while interruption of corticotropin infusion at supramaximal stimulating doses evoked a delay in the fall-off of adrenal secretions. It is suggested that the longer latent periods before 11-OHCS output decline with interruption of high rates of ACTH infusion is the result of prolonged adrenocortical stimulation. A depression of corticoid output in those cases would not become apparent until the circulating ACTH level had fallen below that which produces maximal adrenal steroid output.

Comments of Adrenal Inactivation of ACTH

A reasonable possibility for loss of ACTH activity might appear to be the result of stimulation of and degradation by the target organ, the adrenals. Some loss of corticotropic activity has been reported with exposure of ACTH to isolated rat adrenals, and ACTH has been shown to be concentrated to some extent by rat adrenals (10, 157). However,

the pituitary hormone has been shown to disappear with equal rapidity when injected into both intact and adrenalectomized rats (79).

While blood flow may be high per unit weight of adrenal tissue, total adrenal blood flow is insufficient to accommodate the volume of blood per unit of time to account for the rapid disappearance of ACTH. This can be demonstrated by calculating the $T_{1/2}$ for ACTH assuming the most optimal circumstances: 100% efficiency of peptide removal by the adrenals, inactivation by two intact adrenals and maximal adrenal blood flow.

A half-life for ACTH resulting from adrenal inactivation can be calculated from the classic equation for exponential decay as discussed by Dobson and Jones in reference to liver blood flow measurement (31). That equation is

$$C_t = C_o e^{-kt}$$

where C_t is the concentration of the reference material at time t and C_o is the concentration at time zero. The constant k is the disappearance constant, and if all of the reference material, ACTH in this case, were removed from the blood during passage through an organ, the adrenals, then k would be the fraction of the blood volume circulating through the organ at any time. The constant k in reference to half-time of disappearance, $T_{1/2}$ is

$$T_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k}$$

or,

$$T_{1/2} = \frac{0.693}{\text{adrenal blood flow/total blood volume}}$$

Assuming a total blood volume in dogs of 1000 ml, a high blood flow of 5 ml/min for each gland and 100% efficiency of ACTH inactivation by the adrenals only, the most rapid ACTH half-life possible would be 70 minutes. Under the conditions studied the mean adrenal blood flow was 3.25 ml/min, which considering inactivation by both adrenals would give a $T_{1/2}$ for ACTH of 107 minutes. It is apparent, therefore, that while the adrenal glands may accumulate and degrade ACTH to some degree, the relatively low total adrenal blood flow is incompatible with a system capable of inactivating ACTH at a rate sufficient to produce a half-life of 3.5 minutes.

Effect of Incubation of ACTH in Blood

The very rapid inactivation of adrenocorticotropic hormone necessitates metabolism of the peptide either in blood or in a system with a large blood flow. Considerable instability of ACTH in blood has been reported (145, 148), and within 5 minutes after mixing, as much as 75% inactivation of incubated ACTH in 80% rat plasma has been

observed (154). On the other hand, Richards and Sayers (157), Geshwind and Li (60), and Syndor and Sayers (181) have demonstrated that incubation of ACTH up to 1 hour in either rat plasma or in whole blood does not result in appreciable loss of activity.

Pincus, Hechter and Hopkins have demonstrated the absence of ACTH inactivation in bovine whole blood and plasma, but when the same ACTH preparations were added to both blood and plasma from humans and rats, 40-70 percent of the trophic activity was lost within one hour (147, 148). A heat labile inactivation factor was suggested when rat and human plasma heated to 50°C for 3-5 hours failed to inactivate added ACTH. Forty to seventy percent inactivation in one hour was again observed when ACTH was added to a mixture of beef blood (which failed to inactivate) and to heated rat blood (which also failed to inactivate). The authors thereby suggested the existence of both heat labile and heat stable factors in rat and human blood, and the absence of the heat stable factor in bovine blood. In further studies, these investigators claimed the separation of the heat stable factor as part of the total extraction of certain ACTH preparations which was evidenced by loss of 50-80% of added ACTH activity when incubated with bovine blood or plasma for one hour.

Inactivation of porcine ACTH by dog blood, plasma, and serum as well as inactivation of endogenous ACTH by human plasma in vitro

has been demonstrated by Meakin, Tingey and Nelson (116). The adrenal response to ACTH incubated in whole dog blood demonstrated a decline of 17-OHCS output to the control value in about 160 minutes. From these data a very rough half-life for ACTH of about 30 minutes was estimated. Exposure to plasma and serum showed inactivation of ACTH at a somewhat slower rate, the corticoid output reaching the control value after about 220 minutes. Human plasma showed a similar ability to inactivate endogenous ACTH with an estimated half-time of about 190 minutes, corticotropic activity declining steadily and reaching undetectable levels in seven hours. ACTH activity was found to be stable in human plasma in the frozen state up to 5-6 months.

In a subsequent study, Meakin and Nelson demonstrated the thermal lability of the ACTH-inactivating mechanism by heating dog plasma to 60°C for one hour before incubation with ACTH (115). A temperature dependency of the rate of inactivation was also suggested by the more rapid decline of 17-OHCS following incubation at 0°C. These findings of a thermolabile factor and relation of temperature to rate of reaction of the ACTH-inactivation factor are in keeping with the findings of Pincus (147) and suggest an enzymatic mechanism of catabolism.

In view of the evidence for inactivation of ACTH in blood and to determine if inactivation in blood in vivo may be a major factor in the

rapid disappearance of the hormone, ACTH was incubated in blood and infused alternately with ACTH dissolved in plasma. Blood for incubation was drawn from hypophysectomized dogs and volume-for-volume blood replacement was obtained from intact anesthetized donors. Gelatin-free ACTH was added to both blood and saline just prior to the onset of the respective infusions so as to determine any progressive effect of the two media on hormone activity. Incubation of ACTH throughout the periods of infusion was maintained at room temperature.

Alternate ACTH:blood and ACTH:saline infusions were conducted in 4 animals of which none demonstrated evidence of rapid inactivation of ACTH in blood. Figures 21, 22, and 23 express the results of ACTH-blood and ACTH-saline infusions at rates of 0.6, 0.5 and 0.4 mU/ml respectively. These infusion rates were estimated from rates of 2.5, 2, and 1.7 mU/ml respectively because of discrepancies in activity found in the use of unassayed gelatin-free corticotropin as described in the previous section.

In Figure 21, with the onset of ACTH-blood infusion at 0.6 mU/min the usual rapid rise in 11-OHCS output was observed and this reached a peak of 5.4 μg/min/10 kg after 25 minutes. The buildup half-time of ACTH with the institution of infusion was approximately 3 minutes, which is comparable with the $T_{1/2}$ for disappearance of ACTH from the circulation.

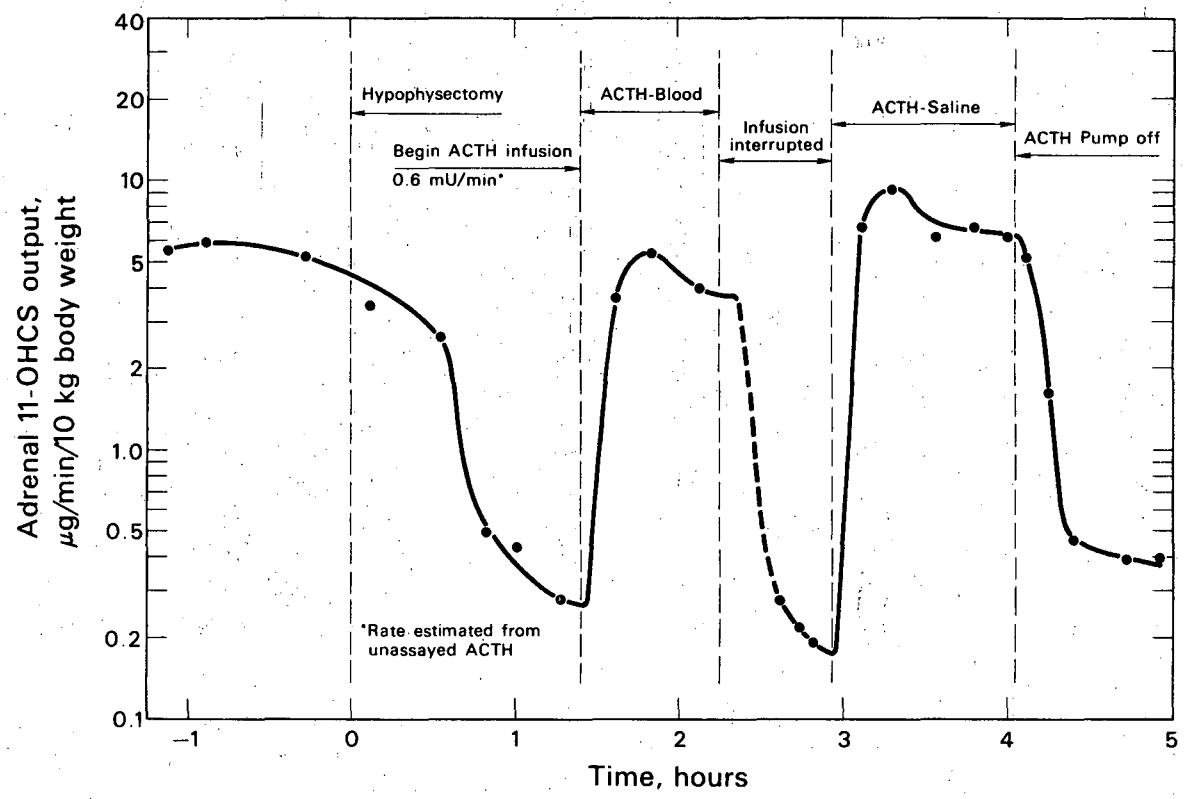

Figure 21. Adrenal 11-OHCS output after hypophysectomy and during alternate ACTH-blood and ACTH-saline infusions (Dog, D-223).

During the last few minutes of the ACTH-blood infusion, the dog encountered respiratory difficulty, whereupon a respirator was connected to the endotracheal tube and infusion was interrupted. This accounts for the drop in 11-OHCS output during that interval.

With the institution of ACTH-saline infusion, the 11-OHCS output rose to a peak of 9.4 μg/min/10 kg within 22 minutes. The $T_{1/2}$ for ACTH buildup in this case was a very rapid 2 minutes.

The 11-OHCS output peak with ACTH-blood infusion was approximately 40% lower than that produced with the ACTH-saline infusion, and the plateau levels showed about the same differences. From these observations it would be tempting to suggest that, as expressed by 11-OHCS output, blood plays a major role in the inactivation of ACTH in vivo.

Whether the problems involved with the respiratory system might be influential in the ACTH-inactivating mechanism cannot be determined from the data at hand, but this aspect should be considered. Adrenal blood flow showed some decline, about 20%, during the ACTH-blood infusion with a rise to the previous flow level during the ACTH-saline infusion. Although this alteration in flow was apparent, it was not considered sufficient to affect steroid output as determined by reports from other investigators and from previous observations.

As determined by the method of constant infusion of labeled chromic phosphate to be described in the next section, liver blood flow

showed a steady decline throughout the ACTH-blood infusion, the respiratory assistance phase, and during the ACTH-saline infusion. The post-hypophysectomy liver blood flow was about 350 ml/min, but by the end of the ACTH-blood infusion this had decreased about 58% to 148 ml/min, and to 107 ml/min, a 70% drop, by the end of the ACTH-saline infusion. If the liver were responsible for the rapid inactivation of ACTH, or if some other organ(s) were the primary inactivator and blood flow were affected, then a decrease in flow should indicate a reduced catabolism of ACTH. In this context, a decline in blood flow should be considered as effecting a reduced rate of delivery of ACTH to the organ and not as producing a reduced nutrition, and thereby impaired functional efficiency, of the organ. As shown in Figure 21, the different 11-OHCS output values produced by ACTH-blood and ACTH-saline infusions might not be a reflection of corticotropin inactivation in the blood, but rather slower metabolism in the organs affected by the reduced blood flow.

Figure 22 shows the effect of ACTH-blood and ACTH-saline infusions at 0.5 mU/min. The rapid decline of 11-OHCS output within 6 minutes after hypophysectomy indicated an ACTH $T_{1/2}$ of about 4.5 minutes. Within a very short time after the onset of ACTH-blood infusion, the 11-OHCS output rose extremely rapidly to 12.5 µg/min/10 kg, and the ACTH $T_{1/2}$ from the corticoid rise was about 1 minute.

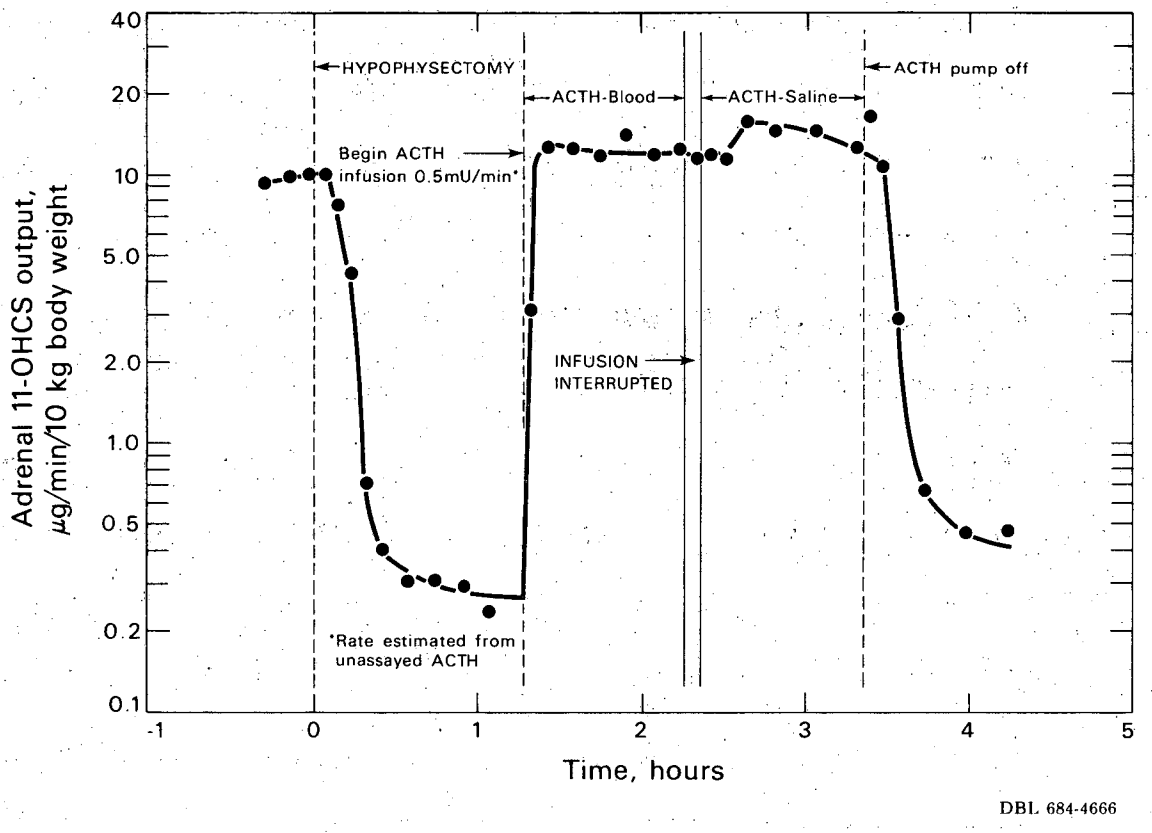

Figure 22. Adrenal 11-OHCS output after hypophysectomy and during alternate ACTH-blood and ACTH-saline infusions (Dog, D-224).

There was a 6 minute interruption in switching from the ACTH-blood to the ACTH-saline infusion, and an ACTH half-life of about 4 minutes was determined from the 11-OHCS decline after the second infusion was stopped.

Although the rate of ACTH infusion in this animal was slightly lower than that shown in Figure 21, 0.5 mU/min versus 0.6 mU/min, it would appear from the maintenance of a high level of 11-OHCS output and the absence of a pronounced overshoot that the infusion at 0.5 mU/min gave a more nearly maximal adrenal stimulation. This is probably due to the individual sensitivity of the animals. It must be considered that at high rates of ACTH infusion sufficient to produce maximal adrenal stimulation, considerable inactivation of ACTH might take place without being reflected by such high rates of adrenocorticosteroid output.

However, since steroid output during the ACTH-blood infusion was maintained at a high level essentially comparable with that produced with the ACTH-saline infusion, it must be concluded that at this level of infusion, incubation of ACTH in blood for the period of an hour does not produce a decrease in physiological activity to account for the rapid half-life of the hormone.

The results of sequential ACTH-saline and ACTH-blood infusions at an estimated infusion rate of 0.4 mU/min are shown in Figure 23.

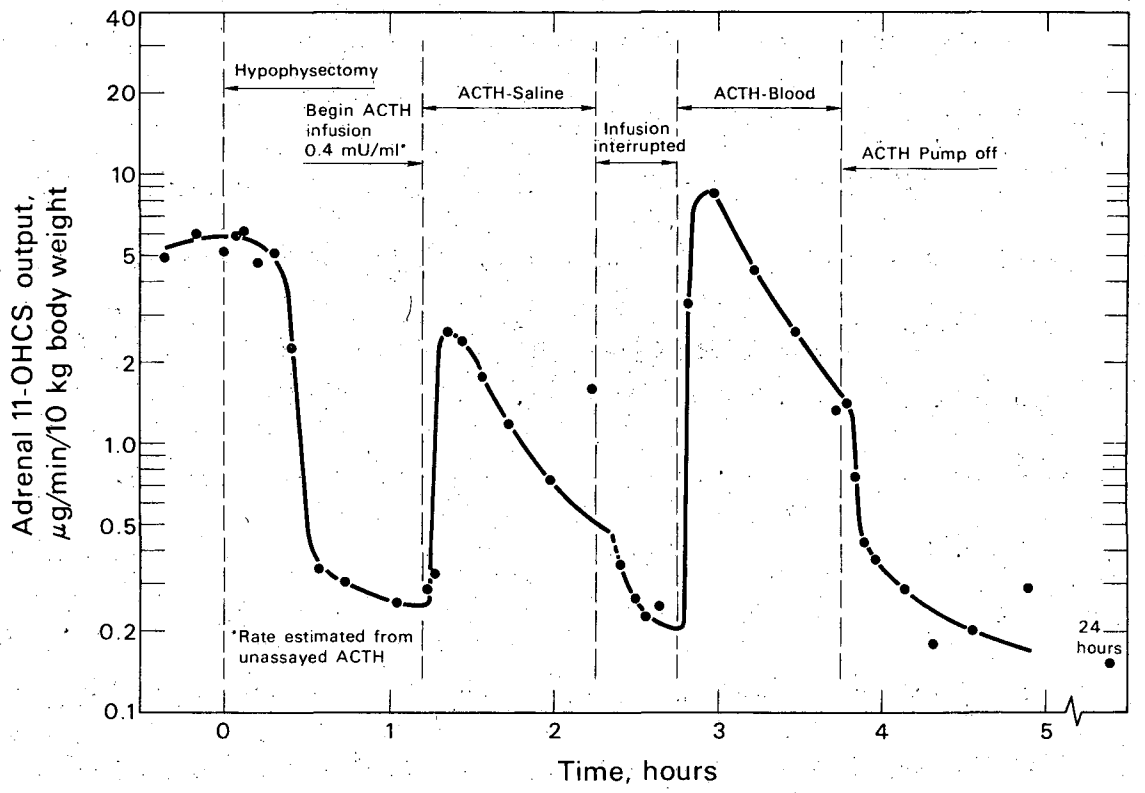

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Figure 23. Adrenal 11-OHCS output after hypophysectomy and during alternate ACTH-saline and ACTH-blood infusions (Dog, D-235).

Following hypophysectomy the decline in 11-OHCS output indicated a $T_{1/2}$ for ACTH of about 3 minutes, reaching an output level of 0.25 μg/min/10 kg after 72 minutes.

11-OHCS output rose abruptly within 3 minutes of the onset of the ACTH-saline infusion and this indicated a $T_{1/2}$ for ACTH of about 2 minutes. The overshoot produced a peak of 2.55 μg/min/10 kg in 9 minutes, but rather than declining to a plateau, the corticoid output continued to decrease.

Following interruption of the ACTH-saline infusion and mixing of ACTH for the ACTH-blood infusion, the drive connection of the infusion pump did not engage, causing a lapse of 30 minutes between the two infusions. During that time the decline of steroid output characteristic of interrupted infusion became apparent, but this was of short duration and of small interval due to the low 11-OHCS output at the time of interrupted infusion.

With the onset of the ACTH-blood infusion, the corticoid output rose abruptly to a peak of 8.5 μg/min/10 kg in about 10 minutes, approximately 3.5 fold greater than that produced with the ACTH-saline infusion. The $T_{1/2}$ for the ACTH buildup with the ACTH-blood infusion was about 1.5 minutes. The corticoid output continued to decline without a plateau throughout this infusion also. After termination of infusion, the 11-OHCS output fell exponentially, indicating an ACTH half-life of

about 2.5 minutes. An adrenal blood sample obtained 24 hours later registered a plasma 11-OHCS output of 0.15 μg/min/10 kg.

The phenomena of the overshoots in Figure 23 - slower decline of corticoid output and absence of plateaus with both the ACTH-saline and ACTH-blood infusions - are peculiar to this experiment and to the peripheral infusion in Figure 27. The higher peak of 11-OHCS output with the ACTH-blood infusion strongly indicates that blood degradation of ACTH activity is not a major catabolic factor, although the reason for the wide difference in the peak values cannot be explained.

The similarity of the 11-OHCS output responses during the two infusions, with the exception of peak levels, is difficult to interpret from the existing information. Adrenal blood flow measurements showed a gradual decrease throughout the two infusion periods, about 30%, which at higher blood flow levels is not sufficient to affect steroid secretion. The constancy of the peripheral blood activity with the infusion of $CrP^{32}O_4$ throughout the post-hypophysectomy period, the two infusion periods, and the interval of interrupted infusion indicates that liver blood flow and probably blood flow in other tissues was stable throughout.

The possibility of leakage somewhere in the infusion system was considered. However, the very close similarity in the two corticoid responses and failure to establish an equilibrium between the rate of

infusion, rate of inactivation and rate of leakage essentially negate this prospect. In addition, the existence of an infusion leak would not evoke a decline in corticoid output suggesting a progressive inactivation of ACTH, but would rather establish a lower, constant level of ACTH stimulation. It is also possible that plateaus may have occurred at times beyond that at which the infusions took place, but observations in both this study and by other investigators do not support this speculation. In a review of the procedure, no differences in the methodology from this to other experiments could be found. The similarity of responses during the infusions suggests that the mechanism or mechanisms by which ACTH activity was degraded must be inherent in the animal itself.

It is quite possible that upon standing in whole blood ACTH undergoes degradation of physiological activity at a slow rate. However, in view of the data presented in this study and lack of supportive evidence to the contrary, it must be concluded that blood cannot be the primary system by which the adrenocorticotropic hormone disappears from the circulation with the rapid half-life of between 2.5 and 5.5 minutes.

While the mechanism of inactivation of ACTH in any tissue remains obscure, certain aspects of the degradation process believed to take place in blood have been determined. Meakin and Nelson found

that addition of L-cysteine to the ACTH incubation medium at 37°C inhibited the corticotropin-inactivating system in plasma as evidenced by a more rapid rate of decline of steroidogenic activity in "ACTH plus plasma" than the decline with "ACTH plus plasma plus cysteine" (115). These authors, in noting the decreased inhibition with L-cysteine, considered the possibility of a process similar to thiol reversible inactivation of ACTH by oxidation with hydrogen peroxide or ferricyanide (25). The oxidation process appears to be pH dependent, being more effective in alkaline than in acidic solutions, whereas reduction by thiols is more effective at acidic pH values. Dedman, Farmer and Morris contended that only five residues of the portion of the ACTH molecules essential for biological activity (serine, methionine, tyrosine, tryptophan and histidine) are expected to be affected by the reversible oxidation-reduction phenomenon, but further inquiry failed to localize the site of inactivation (25). The inactivation of corticotropin by oxidizing agents is further complicated by the fact that this peptide contains no detectable disulfide or sulfhydryl group (7).

White and Gross have shown that porcine ACTH (corticotropin-A) is split by incubation at 37°C and pH 6.0-7.75 with bovine fibrinolysin (200). Lysis was demonstrated after arginine in the 8 position and after lysine in position 15 with a complete loss of physiological activity. The action of fibrinolysin on corticotropin-A is reportedly similar, but not

identical, to the proteolytic action of pepsin, and it has been suggested that the mechanism of proteolysis with fibrinolysin is responsible for the inactivation of ACTH by blood in vitro (200). Through personal communication with Pincus, White and Gross asserted that the heat-labile ACTH-inactivating component of blood has been traced to blood factor III, which contains the precursor form of fibrinolysin.

Inhibition of ACTH in human plasma, as determined by tracer quantities of I^{131} substrate, has been demonstrated by proteolytic enzyme hydrolysis by Mirsky, Perisutti and Davis (120). Human blood contains an inactive proteolytic enzyme related to fibrinolysin, plasminogen, which when activated, possibly by various noxious agents in vivo, is converted to its active form, plasmin. While no degradation of insulin was noted in "native human plasma" in vitro, in streptokinase-activated plasma, in streptokinase-activated plasminogen, or in purified human plasmin, approximately 20% of added ACTH was degraded in about 2 hours in the latter three preparations. Considerable degradation of glucagon and somatotropin was also noted. Meakin and Nelson (115) contended that because only about 10% of Mirsky's ACTH preparation showed degradation in 2 hours in plasma alone (120), this type of inactivation was inconsistent with their observed loss of activity in a comparable time. Mirsky et al. asserted, however, that their results were consistent with findings of ACTH inactivation by human plasma in

spite of the relatively low rate of peptide destruction by plasma alone. This they attributed to the relatively large dose of corticotropin added to a small volume of plasma; approximately 5 units of ACTH incubated with 0.5 ml plasma resulted in the degradation of an equivalent of 0.1 - 0.2 units in 2 hours (120).

As determined by adrenal ascorbic acid depletion of prednisolone treated, nonhypophysectomized rats, inactivation of ACTH in human plasma has been shown to be enhanced with increasing doses of added streptokinase, similar to results observed with caseinolysin (206). Less streptokinase was necessary for full ACTH degradation when the euglobulin fraction was used instead of whole plasma, and some plasmin inhibitors reduced the degradation of ACTH in plasma activated with streptokinase.

The difference in rates and extent of inactivation of corticotropin between the slow degradation in "native" whole blood or plasma and plasma activated with streptokinase is suggestive that hydrolysis by plasmin may not be the inactivation mechanism employed with in vivo systems. In addition, it has been pointed out that conversion of plasminogen to plasmin by streptokinase is specific for human blood, and doses of streptokinase which promote this conversion in human plasma are without effect in other species (120). In view of the evidence for a thermally sensitive system of corticotropin inactivation in blood

and the peptide nature of the hormone, a mechanism of hydrolytic enzyme degradation similar to the plasminogen-plasmin scheme is possible with an activator other than streptokinase initiating the conversion in species other than, and possibly including, man.

Masking of the physiological activity of ACTH by combination with another protein moiety has been suggested by Fekete, who demonstrated inhibition of adrenal ascorbic acid depletion with administration of protamine sulfate prior to ACTH injection in rats (46). Further electrophoretic and chromatographic studies, however, showed that ACTH would have to have a higher molecular weight to form the predicted protamine complex or that protamine would have to exert its in vivo inhibition by complexing with and hydrolyzing a carrier protein of ACTH (47).

Hepatic Relationship to ACTH Inactivation

Effect of Hypophysectomy on Liver Blood Flow

Because of the very rapid half-time of ACTH and in view of the exclusion of blood itself as the primary inactivator, the liver with its large blood flow and high metabolic activity would appear a logical site for catabolism of the pituitary hormone. In beagles it has been demonstrated that colloidal $CrP^{32}O_4$ has a half-time in the circulation of 1.19 minutes with a standard deviation of 0.23, and an average of 97% of the

chromic phosphate was found in the liver and spleen (30). As determined by such an efficient clearance of the colloid in a single passage of blood through the liver, the hepatic circulation would be quite capable of delivering ACTH at a rate sufficient to account for a $T_{1/2}$ of 3.5 minutes.

It is of interest, in addition, to determine the effect of hypophysectomy on liver blood flow, since if the flow were affected, e.g., reduced substantially, the rate of ACTH inactivation would be slower than that observed after interruption of exogenous ACTH infusion. As described in the methods section, during constant infusion of chromic phosphate an equilibrium concentration is established in the blood when the rate of infusion becomes equal to the rate of removal according to the equation,

$$C = \frac{I}{kV}$$

where I is the rate of infusion, V the volume of distribution and k is the disappearance constant (29). With a high efficiency of clearance kV is proportional to the blood flow, and any change in organ blood flow establishes a new equilibrium concentration. Thus, a decrease in blood flow would be reflected in an increase in peripheral blood activity, and an increase in flow would produce a decrease in activity.

Liver plasma flow may be estimated by the equation,

$$LPF = \frac{I}{C_{Eq}}$$

where I is the rate of infusion of chromic phosphate and C_{Eq} is the equilibrium concentration (32, 33).

Liver blood flow as indicated by constant infusion of chromic phosphate was measured in 8 dogs after hypophysectomy. The recorded values in Figure 24 indicate mean blood flows in successive 10 minute periods. The mean of the liver blood flow measurements within the 20 minute period before hypophysectomy was 563 ml/min/10 kg, SEM 20. This is somewhat higher than liver blood flows of 460-508 ml/min/10 kg calculated from liver plasma flows of Dobson et al. (33) (LPF = 254 ml/min/10 kg) at estimated hematocrits of 45-50%. The latter determinations, however, were not affected by surgical manipulation.

Within 10 minutes after hypophysectomy there appeared a significant decrease in liver blood flow to a mean of 502 ml/min, SEM 17.5, but during the next 35 minutes the mean flow levels showed an increase. Between 50 and 60 minutes after hypophysectomy liver blood flow had increased to 666 ml/min, an elevation of about 18%.

Assuming that the liver is the primary organ of ACTH inactivation, it is possible to speculate on the effect of an increase in liver blood flow on the rate of disappearance of ACTH based on the equation,

$$T_{1/2} = \frac{0.693}{k}$$

When all of the material is removed from the circulation during a single

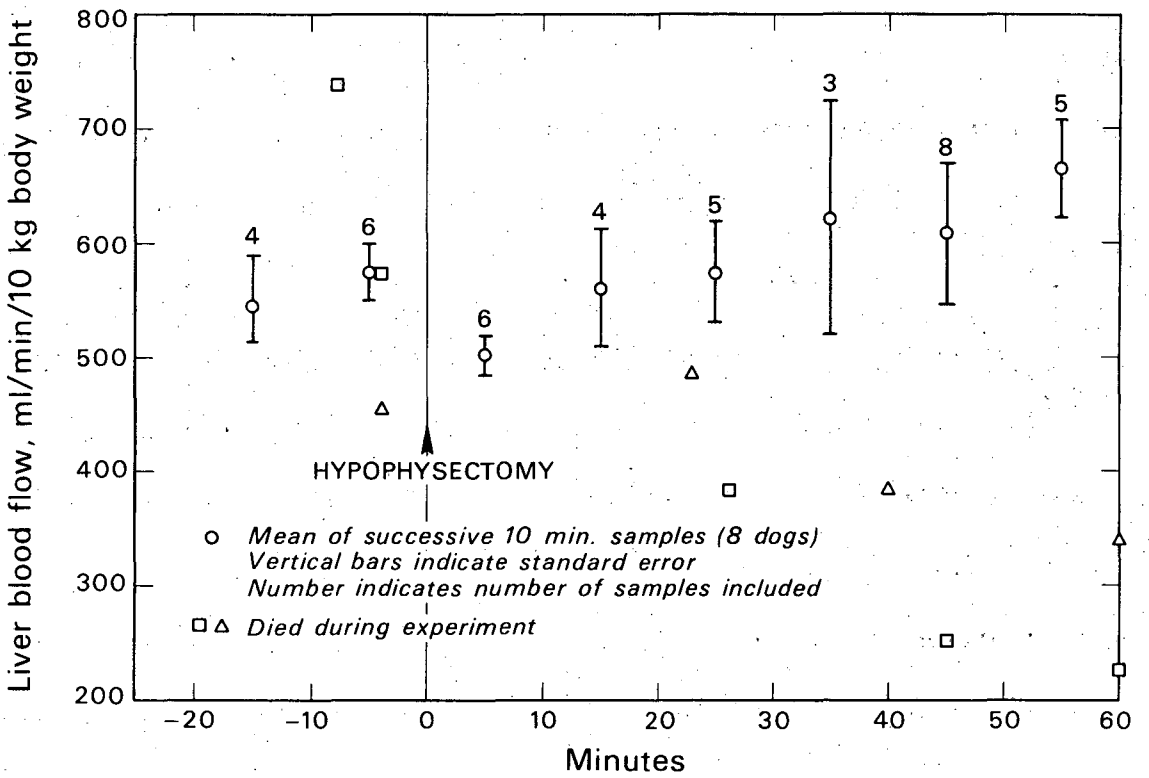

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Figure 24. Effect of hypophysectomy on liver blood flow.

passage of blood through the liver, e.g., essentially 100% removal of chromic phosphate, the disappearance constant k is a measure of the fraction of blood perfusing the organ at any time (5). However, if the liver does not completely remove a material from the blood during a single passage, hypothetically ACTH, then k is the combination of two constants, n, the measure of the efficiency and k, the fraction of the blood volume perfusing the organ at any time (5).

Based on the half-time of 1.19 minutes for chromic phosphate in beagles (30) and the mean $T_{1/2}$ of 3.5 minutes for ACTH determined in this study, the efficiency factor, n, would be 0.34. An increase in liver blood flow from the pre-hypophysectomy mean of 563 ml/min to 666 ml/min an hour after hypophysectomy would produce a somewhat faster half-life value of 3 minutes as compared with a pre-hypophysectomy value of 3.6 minutes. Thus, with the decreases in liver blood flow shortly after hypophysectomy and the increase over the next hour as shown in Figure 24 assuming the liver to be the primary inactivator of ACTH, the alteration in the rate of ACTH inactivation would not be significant, and the $T_{1/2}$ values would fall within the 95% confidence limits of 2.5 - 5.5 minutes.

Whether blood flow through other organs is affected by hypophysectomy and interrupted influence of endogenous ACTH is uncertain. However, Monos, Koltay and Kovach found no substantial alteration of

blood flow through the abdominal organs and posterior part of the body of dogs following hypophysectomy (122).

The liver blood flows of two dogs which died early to mid-way during the experimental procedures are also shown in Figure 24. Within an hour after hypophysectomy the liver blood flows had fallen to 340 ml/min and 225 ml/min, which were decreases of 25% and 60% from their respective pre-hypophysectomy levels. Again assuming the liver as responsible for corticotropin degradation, decreased liver blood flows to these levels would evoke slower ACTH half-times of 4.6 minutes and 9 minutes respectively. The causes of death in these two animals were not determined, nor was the reason for the concurrent drop in liver blood flow deduced. Blood pressure measurements were not recorded.

Portal Infusion of ACTH

To test the extent of inactivation of ACTH by the liver in vivo, ACTH in saline was infused alternately into the peripheral circulation and into mesenteric tributaries of the portal vein in 5 animals. During the infusion of ACTH-saline into one system, saline alone was infused into the alternate system. In none of the cases studied was there an indication of loss of corticotropic activity sufficient to produce a half-life of 3.5 minutes for ACTH.

Figure 25 shows the effect of alternate portal:peripheral ACTH infusion on the output of 11-OHCS after hypophysectomy. Two hours after hypophysectomy infusion into the portal system produced a rapid increase in 11-OHCS output to a peak level of about 4.2 μg/min/10 kg.

A transient interruption of ACTH stimulation was indicated by a drop in 11-OHCS output during the switch from the portal to the peripheral infusion. This may have been caused by backing of blood into the cannula while switching from the saline to the ACTH infusion. The high corticoid output during the peripheral infusion was about 3.8 μg/min/10 kg. Only a gentle rise in output to 3.2 μg/min/10 kg was produced by the second portal infusion. It was determined that the steroid decline near the end of the peripheral infusion and into the next portal infusion was probably associated with a diminished adrenal blood flow, which fell to very low levels during that time. It was apparent, however, that the 11-OHCS output produced with portal and peripheral ACTH infusions were comparable and nowhere suggestive of major degradation of ACTH upon initial passage of the hormone through the liver.

These findings were supported by observations of 11-OHCS output with alternate peripheral and portal ACTH infusions shown in Figure 26. With the onset of peripheral infusion the 11-OHCS output rose rapidly from 0.1 μg/min/10 kg to a peak of 2.6 μg/min/10 kg within 16 minutes.

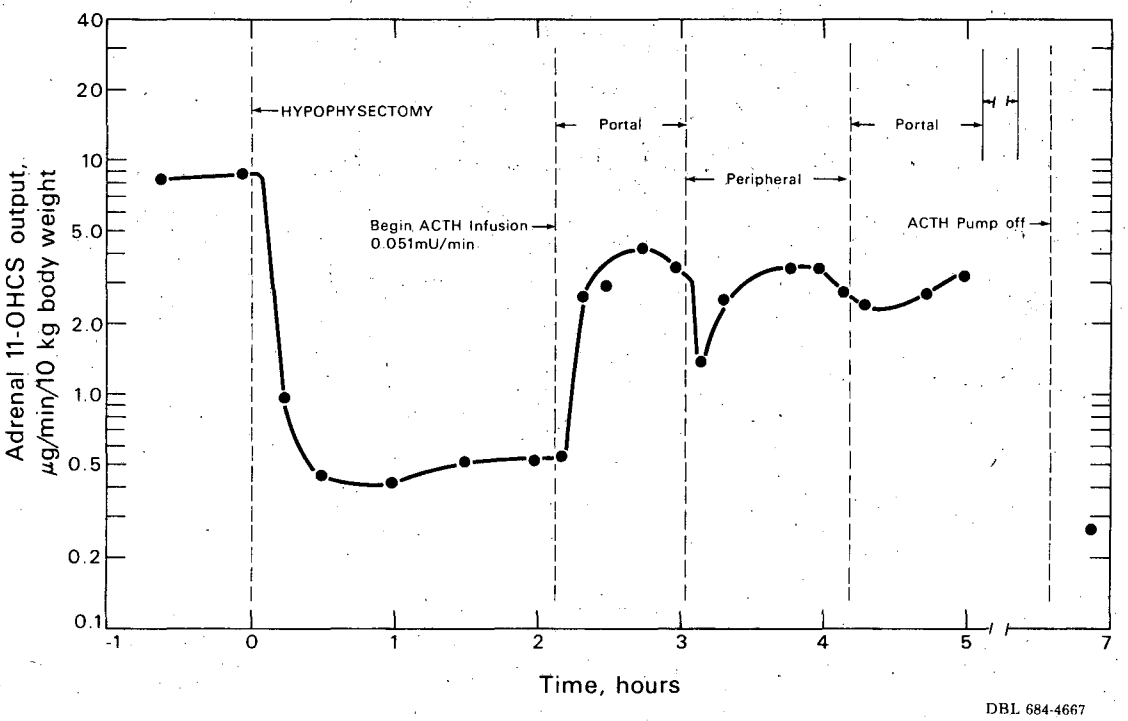
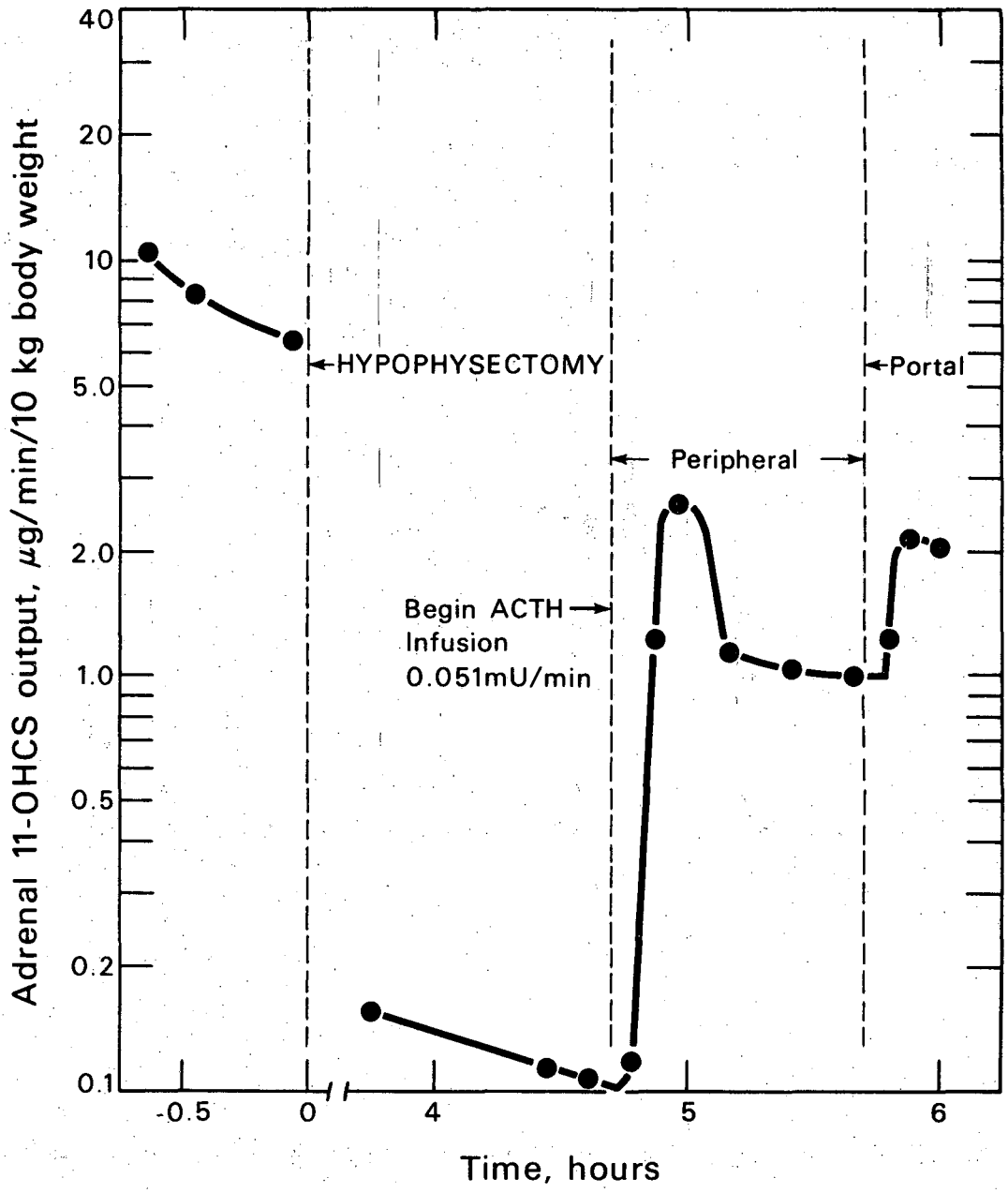


Figure 25. Adrenal 11-OHCS output after hypophysectomy and during alternate portal and peripheral ACTH infusions (Dog, D-197).



DBL 684-4662

Figure 26. Adrenal 11-OHCS output after hypophysectomy and during alternate peripheral and portal ACTH infusions (Dog, D-217).

This rapid buildup described an ACTH $T_{1/2}$ value of about 2 minutes. The overshoot response was quite distinct and fell to an 11-OHCS plateau of about 1 μg/min/10 kg after 45 minutes. When the ACTH infusion was switched to the peripheral circulation, the output level again rose rapidly to a peak of 2.1 μg/min/10 kg within 14 minutes. After that time the output readings became quite erratic, and a corresponding irregularity and extreme decline in adrenal blood flow were observed.

The peak 11-OHCS output values of 2.6 and 2.1 μg/min/10 kg produced with peripheral and portal ACTH infusions were within such close proximity to assume essentially the same magnitude of corticotropin influence on the adrenals in the two cases. Unless the plateau after the portal overshoot would have been of a significantly lower level than that during the peripheral infusion, and from previous observations this would not be likely, then it must be concluded that infusion of ACTH through the liver did not result in any greater inactivation of the polypeptide than did infusion into the peripheral circulation.

The net observation from dogs subjected to alternate peripheral and portal ACTH infusions was that none demonstrated significantly more rapid inactivation when ACTH was infused through the portal system of the liver than when infused through the peripheral circulation. Some of these experiments not shown in graphic form were plagued with various

difficulties, but the observations with respect to corticotropin inactivation were quite consistent. Peripheral-portal-peripheral ACTH infusions (0.05 mU/min) in one animal induced 11-OHCS outputs of approximately 2, 3.2 and 3.4 μg/min/10 kg respectively. One dog died during the latter part of the second infusion but registered respective corticoid outputs of 2.9 and 2.7 μg/min/10 kg with peripheral and portal ACTH infusions (0.6 mU/min). Another dog responded to peripheral graded rates of ACTH infusion with step increases of 11-OHCS output and produced outputs of 13.5 and 10.5 μg/min/10 kg with submaximal adrenal stimulating doses of ACTH (1.2 mU/min) infused peripherally and portally. The pre-hypophysectomy, surgical 11-OHCS output in the latter case was 14 μg/min/10 kg.

The findings of this study, that ACTH was not inactivated in vivo in the liver to an extent sufficient to account for the rapid disappearance half-time of 3.5 minutes, are in keeping with the findings of Meakin, Tingey and Nelson (116). In that study ACTH activity was determined by adrenal corticoid output of 10 minute adrenal blood samples following 5 milliunit injections of porcine ACTH. Although animal weights were not specified, it was estimated that the resultant blood ACTH concentration after injection of 5 mU of ACTH in Meakin, Tingey and Nelson's study would be essentially comparable to that during constant portal ACTH infusions in this study. Using the adrenal response to the femoral vein

injection as 100 percent, the corresponding response after injection through the liver was 92%, through the leg via the femoral artery 87%, through the kidney 85%, and through the adrenal gland via the adrenal artery 84%. The high percentage of activity and the wide range of standard deviations in each determination indicated that there existed no preferential binding of ACTH by these tissues.

Lack of binding of ACTH by liver tissue was also demonstrated by Richards and Sayers (157). Five minutes after administration of extracted rat ACTH into rats, 40% of the injected dose was measured in the extracellular fluid and 20% in the kidney. After 15 minutes only a very small amount was found in the extracellular fluids and 15% in the kidneys. The liver contained no measurable ACTH at either 5 or 15 minutes. Although not mentioned by the authors, the absence of measurable ACTH in the liver might also be interpreted to indicate catabolism of ACTH in that organ. In spite of the large concentration of the hormone in the kidneys, no ACTH activity was demonstrated in the urine of these animals.

Geshwind and Li found marked inactivation of ACTH incubated with liver and kidney slices, with diaphragm, and with homogenates of liver, kidney and adrenal (60). In comparing degradation properties of liver and kidney slices, the latter showed the greater capacity. Liver homogenates were more effective at reducing ACTH activity than liver

slices, and, while considerably reduced, heating of the liver homogenates did not completely abolish the ability of the liver to inactivate ACTH.

Inquiring into the nature of the residual inactivation potencies of heated tissues ("heated" tissues were placed in a boiling water bath for 15 minutes before incubation at 37.4°C), the same authors found that about 15% of the corticotropic activity could be recovered by eluting heated diaphragm tissue with strong alkali or acid (60). Evidence for a binding-type of inactivation was also presented in the recovery of corticotropic activity upon washing of liver homogenates incubated with ACTH at 38.1°C and 6°C. A greater amount of activity was recovered after incubation at the lower temperature.

In view of suggestions of both an adsorption or binding mechanism and a heat-sensitive component in ACTH inactivation, Geschwind and Li separated the particulate fractions of liver cells by differential centrifugation (60). The supernatant showed the greatest inactivation ability, with considerable potency residing in the mitochondria and to a lesser extent in the nuclei and microsomes. Total unheated homogenates from which the fractions were prepared almost completely inactivated ACTH, and no combination of particulate fractions and supernatant was capable of duplicating this degradation capacity. It was also determined that the catabolic mechanism involved produced more rapid inactivation of peptide fractions than of protein.

From the discussion above, it will be noted that greater degrees of ACTH inactivation were produced upon exposure of the hormone to tissue homogenates than to tissue slices. In conjunction, Sayers observed that as long as the integrity of the tissue was maintained during incubation of ACTH with pituitary, the hormonal activity was not reduced, but when the hypophyseal cells were crushed and incubated with ACTH, hormone activity disappeared.

From the available data it must be concluded that the adrenocorticotropic hormone is not rapidly metabolized in the liver in vivo to a substantial degree in spite of reports of inactivation by liver slices in vitro and considerable degradation of the hormone by crushed cells from liver and a variety of other tissues.

Effect of Renal Infusion of ACTH

The implication of the kidney in the disappearance of ACTH has been expressed by the work of Richards and Sayers cited previously (157). Five minutes after the administration of ACTH, 20% of the dose was found in the kidneys of rats, and 15% was still resident after 15 minutes. However, no corticotropic activity could be detected in the urine of the test animals. Cats, Meyer and Kassenaar injected I^{131}-labeled ACTH into rats and measured a considerable concentration of activity in the kidneys (24). This activity level did not change in early

samples but showed a decline at 60 and 120 minutes. The bladder registered a gradual increase in activity as did thyroid tissue to a lesser extent.

The authors concluded that most of the I^{131}-labeled compounds concentrated in the kidney were metabolized and "excreted" into the circulation rather than being excreted in the urine (24). Although the products were not identified, chromatograms showed that both iodide and some I^{131}-containing compounds were excreted in the urine. In view of the high concentration of dehalogenase in the kidney, the investigators speculated that these compounds were probably iodotyrosine-containing peptides rather than iodotyrosine as such. Comparative chromatograms indicated that if unchanged corticotropin were excreted in the urine the amount must have been very small.

In an earlier work Cats and Kassenaar demonstrated that the disappearance of activity from the blood of rats following a single dose of I^{131}-labeled corticotropin was slower in kidney-ligated than in non-ligated animals (23). Hormonal activity did not appear to be a determinant in the renal accumulation of I^{131}, since there were no differences in the uptake of labeled corticotropin, an inactive hypophyseal protein and a placental protein (22).

It has been emphasized by Cats and Kassenaar, however, that "the decrease of radioactivity in the blood during the first period after

injection cannot be used as an index for the rate at which the administered corticotrophin is metabolized in the organism" (23). The essential problems in drawing conclusions from the use of labeled ACTH are the questionability of label incorporation in the active portion of the molecule and the extent to which hormonal activity is associated with accumulation of radioactivity by an organ. As stressed by Sonenberg in the use of labeled pituitary hormones, the chemical character of the radioactivity which localizes in different tissues has not been identified and proof of association of detected radioactivity with hormonal activity is lacking (174).

Upon assay for ACTH in surgically stressed and hemorrhaged dogs, Gold et al. found the kidneys tested to contain less than 4 mU/kidney (63). The adjusted ACTH content for two kidneys was determined to be sufficient to produce only a transient maximal corticoid output in hypophysectomized dogs. It was concluded to be unlikely that the kidneys possess an extra-pituitary storage capability for ACTH to be released into the circulation in emergency situations. ACTH content of the urine was not determined in this study.

In contrast to the above findings Meakin, Tingey and Nelson recorded no significant difference in loss of hormone activity between ACTH administered into the femoral vein and into the renal artery (116). In addition, Ganong has shown that the response of the adrenals to ACTH

doses of 2, 5, and 10 mU did not differ between nephrectomized and kidney-intact hypophysectomized dogs (56).

Figure 27 shows the effect of ACTH infusion into the renal artery of a hypophysectomized beagle. While data of adrenal 11-OHCS output during renal ACTH infusion were obtained from only two animals, neither showed measurable inactivation of ACTH by the kidney. The infusion rate of 0.4 - 0.5 mU/min in Figure 27 was calculated from a rate of 1.6 mU/min for unassayed gelatin-free ACTH as described previously. With the onset of infusion the 11-OHCS output rose abruptly within 3 minutes and attained an overshoot peak of 14.5 μg/min/10 kg at 34 minutes.

Upon switching to the renal infusion, the corticoid output again attained a peak of 14.5 μg/min/10 kg in 19 minutes. The second peripheral ACTH infusion produced an output peak of 13.5 μg/min/10 kg about 7 minutes after switching from the renal infusion.

As demonstrated by the high 11-OHCS output during the three phases of ACTH infusion, the adrenals were probably secreting corticosteroids at a high but submaximal rate. From past experience ACTH infusion rates of this order have produced submaximal adrenal stimulation. Adrenal blood samples were not obtained for periods long enough to allow establishment of output plateaus, but from the trend of the curves it was estimated that the plateaus for the first peripheral infusion

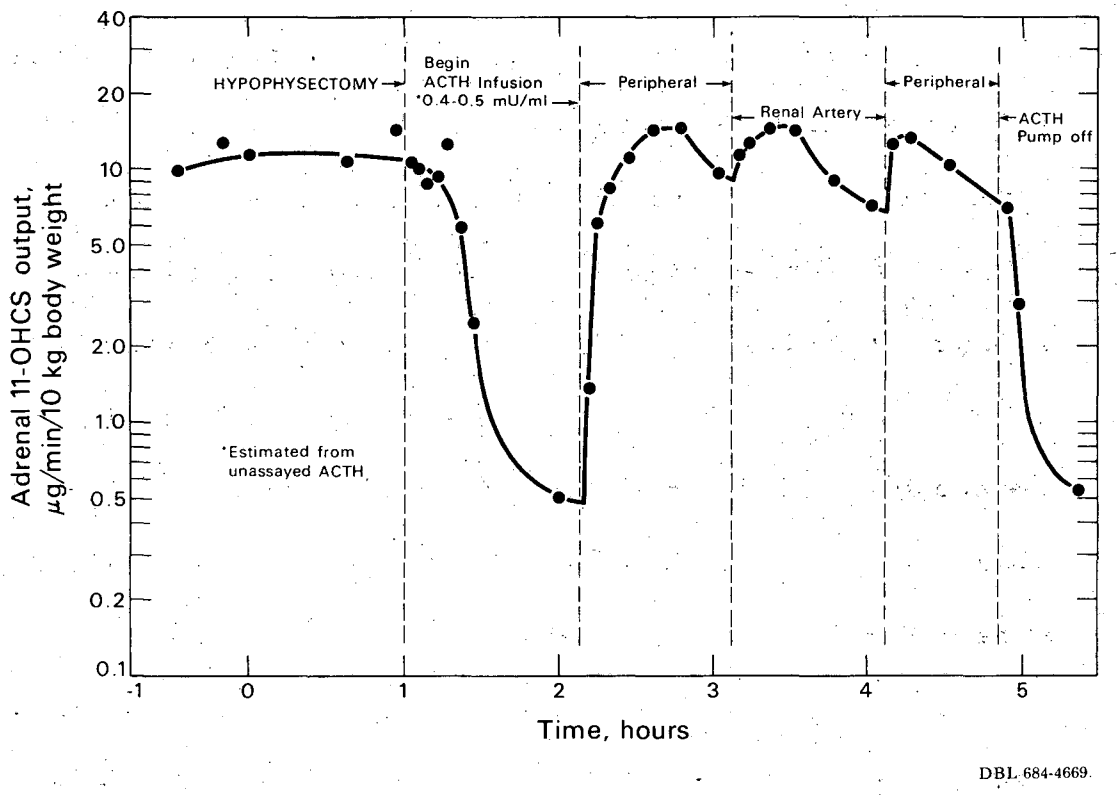


Figure 27. Adrenal 11-OHCS output after hypophysectomy and during alternate peripheral-renal-peripheral ACTH infusions.

and the renal infusion would be about 8 and 6 μg/min/10 kg respectively. This represents a 25% difference between stable circulating levels of ACTH, indicated by 11-OHCS output, with the two infusions. Whether the level produced by renal infusion represents a significant inactivation of corticotropin by the kidney cannot be specifically stated from these data. It must be considered, however, that high rates of adrenal corticoid output may not reflect inactivation of large stimulating doses of ACTH to the same extent as at lower rates of corticoid secretion.

Superimposition demonstrated a remarkable similarity between the shapes of the overshoot profiles during the first peripheral and renal infusions, even though the former produced a peak at a relatively later time. The earlier peak during the renal infusion was probably produced because of the already high level of adrenal corticoid output.

As described in the section on graded rates of ACTH infusion, the existence of pronounced overshoot responses such as those produced with peripheral and renal ACTH infusions in Figure 27 indicates sub-maximal adrenocortical stimulation. As such, the respective peak output readings, though possibly depicting maximal adrenal secretion at their apices, were not flattened as would be the case with absolute maximal stimulating doses. This evidence implies that infusion of ACTH initially through the kidney did not provoke a lower circulating level of the hormone than did infusion into the peripheral circulation.

In spite of reports of accumulation of radioactivity in the kidneys following administration of labeled corticotropin, little is known of the relationship between concentration of isotope and loss of hormone activity. Considering this aspect and in view of observations in Figure 27, equivalent hormone activity with femoral vein and renal artery ACTH infusion (116), and comparable adrenal response to ACTH in nephrectomized and "intact" hypophysectomized dogs (55), it must be concluded that the kidneys are not of primary importance in the catabolic mechanism of ACTH.

SUMMARY AND CONCLUSIONS

The derivation of information concerning the kinetics of adrenocorticotropic hormone inactivation from this study was limited, as in other investigations involving ACTH, to the influence of the hormone on the range of secretion of adrenal corticosteroids. As such, data relating to corticotropin activity were deduced only indirectly, and interpretation of results was complicated by the effects of anesthesia and surgery.

The mean pre-hypophysectomy plasma 11-OHCS concentration in intact beagles was 0.26 μg/ml, and comparable results were recorded for mongrels. A significant factor in the measurement of ACTH activity was the time of day at which blood samples were obtained. Beagles exhibited a pattern of circadian periodicity of circulating 11-OHCS similar to that reported for man, monkeys and mongrels, i.e., diurnally active animals (120, 182, 190). The high mean 11-OHCS level of 0.31 μg/ml plasma occurred at about 8:45 AM with the steepest decline between noon and early evening. The lowest mean steroid level of 0.24 μg/ml occurred at about midnight, and this low level continued until about 4:00 AM. In relation to the present study it would be of interest to investigate the possibility of a circadian periodicity associated with the ACTH inactivation mechanism.

Within 10 minutes after administration of barbiturate anesthesia, the mean plasma 11-OHCS concentration declined from 0.26 μg/ml to 0.11 μg/ml and, in the absence of surgical disturbances, maintained that level for at least an hour. 11-OHCS suppression following anesthesia was considerably more rapid than that after hypophysectomy, suggesting contributing factors other than anesthetic block of ACTH release. The blocking action of barbiturate anesthesia on ACTH release is not overcome by trauma induced by minor surgery but is broken by more extensive surgical maneuvers. Intraperitoneal exposure and lumboadrenal vein cannulation evoked near-maximal adrenal 11-OHCS release.

A two-component pattern of 11-OHCS metabolism after hypophysectomy was demonstrated with a half-life of 76 minutes, 95% confidence limit range of 52-150 minutes, characteristic of the initial phase. Sixty minutes after pituitary ablation, a slower 11-OHCS decline, $T_{1/2}$ = 170 minutes, suggested the movement of adrenal steroids into the circulation from the lymph and extravascular compartments of the "outer pool."

The high mean adrenal 11-OHCS output prior to hypophysectomy, 9.6 μg/min/10 kg, was attributed to trauma induced by adrenal vein cannulation. The pattern of disappearance of ACTH from the circulation after hypophysectomy was characterized by a very rapid half-life of 3.5

minutes, 95% confidence limit range of 2.5 - 5.5 minutes, and a fall of adrenal 11-OHCS output to the order of 0.2 μg/min/10 kg.

Following the initial rapid disappearance of circulating ACTH with hypophysectomy, which accounted for about 90% loss of hormone activity, a slower component of degradation was apparent. The $T_{1/2}$ for this decline was in the range of 45 minutes. This component is also indicative of re-entry of the hormone into the circulation from the "outer pool" and would be comparable to a slow, diminishing rate of corticotropin infusion. Such a shift toward a new equilibrium, even in cases of normal ACTH suppression, may serve physiologically to assist in the maintenance of a relatively stable circulating hormone level.

While adrenal plasma 11-OHCS concentration readings approached the peripheral values after hypophysectomy, the two did not become equal. The slightly higher adrenal 11-OHCS readings suggested adrenal stimulation from ACTH diffusing from the extravascular compartment, "leakage" of steroids from the adrenals, and minute adrenocortical stimulation from residual pituitary cells or from extra-hypophyseal sources.

ACTH infusion rates between 0.01 and 0.2 mU/min evoked increasing adrenal 11-OHCS output patterns which were interpreted as being proportional to something intermediate between the stimulating dose of ACTH and the logarithm of the ACTH dose.

ACTH infusion at 0.05 mU/min was the lowest rate to produce a distinct elevation of adrenal 11-OHCS above the basal hypophysectomy level, and maximal adrenal secretion occurred at corticotropin infusion rates between 0.5 and 0.85 mU/min. 11-OHCS output was not elevated further with ACTH stimulation 20 times greater than that sufficient to produce maximal 11-OHCS secretion.

Graded rates of ACTH infusion produced graded increases in 11-OHCS output. While not apparent at low levels of adrenal secretion, a prominent feature of the 11-OHCS rise with sudden increases in ACTH stimulation was an overshoot response, the time factors of which coincided closely with those described by Urquhart and Li (190). As the rates of ACTH infusion approached high levels, the 11-OHCS overshoot profile became broader, progressively subdued and finally flattened at maximal 11-OHCS output. In view of the absence of the overshoot response at high and low levels of adrenal stimulation, the factors initiating the phenomenon are possibly ACTH-adrenal interdependent rather than resident in the ACTH kinetics alone and may be related to rapid corticoidogenesis and/or the release of corticoid reserve.

An undershoot of adrenal 11-OHCS output was observed in a few cases after incomplete hypophysectomy. This event was similar to the undershoot response produced by abrupt step decreases of ACTH infusion as described by Urquhart and Li (190).

In hypophysectomized dogs, it was noted that with interruption of exogenous ACTH infusion, the latent period preceding the fall of adrenal 11-OHCS output was longer at higher ACTH dose levels.

Amid a spectrum of conflicting reports, observations in this study showed little or no change of adrenal blood flow with decrease in circulating ACTH levels after hypophysectomy. A transient drop in blood flow was initiated prior to ablation of the pituitary, but it was suggested that the suppression of flow might have been provoked by mechanical trauma during cannulation of the lumboadrenal vein.

Loss of ACTH activity and accumulation of radioactivity from labeled ACTH has been reported in rat adrenals (10, 157). However, to be considered as responsible for the primary inactivation of ACTH, an organ must possess a large blood flow. While the adrenals may participate to some extent in the degradation of the pituitary hormone, under optimal conditions they could not produce an ACTH half-time of less than 70 minutes.

In view of maintenance of corticotropic activity during alternate infusion of ACTH incubated in blood and in saline, it was concluded that blood alone cannot be responsible for rapid inactivation of corticotropin. Relatively slow inactivation of ACTH in blood appears to be quite probable. This has been attributed to enzyme activity similar in action to the hydrolysis of peptides by fibrinolysin (200).

Because of its extensive and varied metabolic activities, the liver seemed a logical site for inactivation of ACTH. In addition, the large hepatic blood flow is quite capable of presenting the pituitary hormone to the liver at a rate sufficient to account for the short half-life. However, infusion of ACTH into the portal circulation failed to produce depression of adrenal 11-OHCS output below that elicited by peripheral infusion. Reports of a heat-sensitive, binding-type of ACTH inactivation mechanism exhibited by liver, kidney, and diaphram slices and by homogenates of liver, kidney, and adrenals (60), suggest a slow rate of hepatic ACTH metabolism that could not be detected by methods in this study.

Concentration of radioactivity in kidneys has been reported following administration of labeled corticotropin (22-24); however, valid conclusions of ACTH inactivation are still outstanding pending information relating concentration of radioactivity and loss of hormone activity. No major inactivation of ACTH by kidneys was detected as the result of corticotropin infusion into the renal artery. This conclusion verifies data from other renal infusion studies (116) and observations of comparable ACTH activity in both nephrectomized and kidney-intact dogs (56).

It has been demonstrated that adrenocorticotropic hormone is inactivated in vivo in the dog with a very rapid half-life of 3.5 minutes.

Under the conditions studied, major degradation of the hormone did not appear to reside in the liver or the kidneys. It was also concluded that primary inactivation of ACTH does not take place in blood or in the adrenals.

Failure to demonstrate loss of corticotropic activity in any of the systems most reasonably capable of such action leads to the speculation that perhaps all, or many tissues, may contribute in varying degrees to degradation of the pituitary hormone. Since inactivation has been demonstrated in a number of tissues, this concept is quite plausible. As expressed by Geschwind and Li in noting ACTH degradation in several tissues and in considering the widespread distribution of cathepsin and other proteolytic enzymes, "It would . . . be premature to assume the presence of any specific - for the inactivation of ACTH - enzyme in any tissue" (60). It is therefore suggested that loss of ACTH activity as represented by a half-life of 3.5 minutes is the result of metabolic processes occurring in many tissues, each with a low efficiency, such that significant arterial-venous hormone differences are not detectable (42).

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APPENDICES

Metabolism of Corticosteroids

In addition to a number of papers on the varied aspects of adrenal steroid metabolism, a number of surveys and reviews of accumulated knowledge concerning corticosteroid metabolism are available of which those cited here are but a few (19, 34, 112 vol. 2, 138, 149, 182, 208). The following, while considerably incomplete in many respects, is a brief presentation of the present conceptual status of the metabolism of adrenal corticoids.

In both man and dogs evidence indicates that altered clearance of cortisol during surgery and prior to death is the result of reduced hepatic activity (38, 160, 184). It is probable that the metabolism of cortisol involves reduction of the steroid and then conjugation (160). Metabolism of most of the cortisol involves the reduction of ring A of the cyclopentophenanthrene nucleus by a specific triphosphopyridine nucleotide-linked enzyme, Δ^4-hydrogenase, resulting in the formation of β-dihydrocortisol. The latter is further reduced by a second specific enzyme, 3 α-hydrosteroid dehydrogenase, to tetrahydrocortisol (57, 143).

Some cortisol is converted in the liver to cortisone, which is not synthesized by the adrenals but which is a highly active glucocorticoid and the first to be commercially produced (57). The tetrahydroglucuronide conjugates of both cortisol and corticosterone are freely soluble, enter the circulation, and are excreted in the urine as a part of tubular secretion (57).

In man, corticosterone disappears much more rapidly from the circulation, $T_{1/2}$ = 30-60 minutes, than does cortisol (35, 39, 57, 78, 84, 129, 139, 208). This evidence was based largely on the faster clearance of C^{14}-corticosterone and the more rapid appearance of its glucuronides, in comparison with studies utilizing C^{14}-cortisol. Even though corticosterone is metabolized rapidly, a smaller proportion of its conjugated products in the plasma are removed during passage through the kidneys, indicating a difference in the way the kidneys handle conjugated metabolites of the two corticosteroids (119). The low level of appearance of corticosterone metabolites in the urine appears to be associated with an increased secretion through the bile, but there is no significant difference in the 48-hour excretion of cortisol and corticosterone when both biliary and urinary routes of elimination were considered (119).

Although half-time values were not stated, Nelson and Harding noted that the 17-OHCS levels reached a maximum of 50-200 μg/100 ml

in blood of dogs following intravenous injection of cortisone (129). With the completion of injection the levels then fell to normal in about 60 minutes.

Metabolism of 17-OHCS by the liver is apparently governed by the concentration in the plasma and on the hepatic enzyme activity irrespective of the peripheral requirements for the hormone (143).

The administration of tetrahydrocortisone to terminal patients results in the clearance of the compound comparable with that in normal subjects indicating that the conjugation step is unimpaired (160). The normal rate of clearance of infused tetrahydrocortisol in patients with cirrhosis of the liver also indicates a normal rate of conjugation (140).

The projection of a similar overall mechanism of corticosteroid catabolism in dogs to that in man stems from the work of Axelrod and Miller, who perfused isolated dog livers with hydrocortisone and identified metabolites of saturated 3-hydroxycompounds in mixed chromatograms (3).

The works of Nelson and Harding (131), of Sandberg et al. (161), and of Tyler and co-workers (184) give added emphasis to the importance of the liver in the metabolism of corticosteroids. From BSP tests (bromsulfphthalein) for liver function, it has been noted that post-operative increases in liver retention of BSP were highest in patients who showed the higher elevations of steroid levels (161). The authors

cautioned, however, that the hepatic retention of BSP presents only presumptive evidence that reduced liver function plays a major role in impaired steroid metabolism under these conditions.

In liver function tests involving the infusion of cortisol, it has been demonstrated that while normal patients and patients with liver disease (cirrhosis and hepatitis) exhibited exponential clearance of cortisol with time, the mean corticoid disappearance in patients with liver disease was significantly slower than in normal patients: 244 minutes and 116 minutes respectively (16). In addition, in cases of hepatitis the removal of corticoids from the circulation became more efficient as the severity of the disease decreased. Patients with liver disease excreted less conjugated 17-OHCS in the urine than did normals, while excreting larger amounts of free corticoids (16).

Fedotov and Rynkova have demonstrated a decrease in the retention of 17-OHCS in the livers of irradiated dogs (45). They attributed this to suppressed binding of corticosteroids, depressed enzymatic activity, and to a possible greater utilization of the hormones by body tissues.

Plager et al. found that the volume of distribution of cortisol approximated closely that of the extracellular fluid (150). This suggested that the time for development of equilibrium between the arterial blood and the tissue fluids in the arteriolar and capillary systems and the

diffusion time throughout the available tissue space would tend to lengthen the clearance time. Glenister and Yates determined the virtual volume of distribution of corticosterone in rats to be 45-46 per cent of the total body weight (62).

The disappearance curve of cortisol suggests a single compartment volume of distribution with first order kinetics characteristic of the removal process (208). Nugent, Eik-Nes and Tyler in studies of the suppression of adrenal secretion with dexamethasone and with interruption of cortisol infusion noted that their data did not fit the interpretation of a first order kinetics single compartment model (135). Such a two compartment system would be conceivable if the rate of re-entry of cortisol from the interstitial fluids into plasma, and from any other of the "outer pool," were slower relative to the removal rate of plasma 17-OHCS by the liver (208). In that case, the re-entry would be equivalent to a rather slow, continuing infusion.

Peterson et al. determined a mean volume of distribution of 70 liters in man at the time of estimated complete mixing of intravenously administered cortisol (143). This is a volume comparable with that of the total body volume and implies that hydrocortisone is concentrated in some portions of the body at higher levels than that in the plasma.

Localization of cortisol in the red blood cells has been demonstrated. In human blood , hematocrit 40 per cent, it is estimated that

75 to 80 per cent of the circulating hydrocortisone appears in the plasma with 20 to 25 per cent in the erythrocytes (143). Seventy five per cent of the cortisol present in the plasma was observed bound to non-diffusible elements of the plasma (143).

Holzbauer and Vogt demonstrated a distribution of aldosterone and glucocorticoids between the plasma and cellular components of adrenal venous blood of 4 mammalian species – dogs, rats, guinea pigs and rabbits (87). In dogs, cortisol was found nearly equally distributed between the plasma and cell fractions, while corticosterone was always in higher concentrations, never lower, in the plasma, though this excess was occasionally quite small. The mean plasma-cell concentration ratio of cortisol and corticosterone for 15 dogs was 1.16 and 1.80 respectively, while that for aldosterone was 0.61. In rats, guinea pigs and rabbits, about the same distribution of glucocorticoids was found as in the dog: about 75% of the corticosterone was in the plasma, and cortisol was about equally distributed between the plasma and the cells.

It was demonstrated that the distribution of the 3 steroids, particularly aldosterone, varied independently from dog to dog (87). This suggests one possible explanation for the individual variability of 17-OHCS cited earlier. The association of steroids with blood cells was easily disrupted by washing of packed cells with saline solution, indicating a possible association of the hormones with the cell membranes (87). The

volume of plasma trapped in the packed cells was too small to account for the concentrations in the saline washes. Since the cell fraction includes red cells, white cells and platelets, no conclusions of cell types involved in the steroid association were presented, but it was suggested that possibly all three types are involved.

Radioautographs have demonstrated concentration of topically administered hydrocortisone in the cytoplasm of fibroblasts of mice (35). Within 6 hours after administration, marked granulation and increased epithelioid appearance of the cells occurred, and these appeared to be associated with a greater resistance to fibroblastolysis.

While the functional state of the liver cells appears to be a primary determining factor in the metabolism of cortisol, the implication of changes in liver blood flow has also been expressed. Tyler and co-workers suggested that both functional damage to liver cells and impaired liver blood flow are accountable for elevated 17-OHCS levels following surgery in man (184). Eik-Nes and Samuels, in noting the depression of cortisol metabolism in cold-stressed, anesthetized dogs, suggested the influence of decreased liver blood flow, although the effect of lowered body temperature on optimal enzyme activity was also considered (38).

Yates and Urquhart, however, emphasize that minor changes in liver blood flow have no influence on alteration of adrenal corticoid

metabolism, and only when the change in blood flow is extreme is steroid metabolism significantly altered (208). Glenister and Yates maintain that if all corticosterone were extracted in a single passage of blood through the liver in rats, the disappearance would still follow first order kinetics even if blood flow, rather than hepatic enzyme activity, were rate-limiting (62). This has been demonstrated by the removal of virtually all of the injected colloidal chromic phosphate in a single passage of blood through the liver of dogs, $T_{1/2}$ = 1.2 - 1.4 min, and the disappearance followed first order kinetics (30, 31). The $T_{1/2}$ for corticosterone, 22.2 minutes, SEM 1.55, in male rats and 12.7 minutes, SEM 0.83, in females, and data from intact and perfused livers indicate that the liver does not extract all of the hormone in a single passage. The authors thereby submitted that the half-life of corticosteroids is independent of liver blood flow except in instances when the hepatic flow approaches zero (30, 31).

The level of Δ^4-steroid dehydrogenase activity in the liver appears to be the main determinant in the disappearance of corticosterone in rats (62). In addition, reduction of the volume of hepatic tissue by partial hepatectomy in rats, i.e., reduction of the amount of available enzyme, had demonstrated a diminished capacity for hepatic inactivation of adrenal steroids (192). From evidence in cirrhotic men, Peterson concluded that although no diminution in the conjugation of tetrahydrocorticoid

metabolites of cortisol was seen, hepatic activity of the specific TPNH-dependent enzyme catalyzing the reduction of the 4, 5 double bond of cortisol was evident (140). In association with this, there was marked reduction in the rate of synthesis of corticosterone and cortisol in cirrhotics. However, these patients were considered as "eucorticoid" with respect to plasma 17-OHCS levels due to the diminished catabolism of corticosteroids.

The demonstration of hepatic enzyme activity as a rate-limiting factor in the inactivation of 17-OHCS has prompted suggestion that the hepatic enzyme system determines the rate of secretion of ACTH in undisturbed animals (62, 209). The basis for this conclusion stems from the negative feedback control of corticosteroid secretion by which a high 17-OHCS level produces suppression of the release of ACTH. This reduced stimulus to the adrenal cortex along with catabolic activity of the Δ^4-steroid dehydrogenase system combine to reduce the corticosteroid level and to maintain this within normal resting limits. According to the above hypothesis of corticotropin control, it would be reasonable then to assume an influence of the thyroid on the release of ACTH, since thyroid hormones accelerate the removal of biologically active corticosteroids from the blood of rats (209). The total capacity of the liver for inactivation of cortisone by ring A reduction is increased in hyperthyroid rats and reduced in hypothyroid animals.

TABLE III

PERIPHERAL PLASMA 11-OHCS CONCENTRATION AND HEMATOCRIT IN MONGREL DOGS

	Weight (kg)	Hct (%)	11-OHCS (μg/ml)
	23.2	53.2	0.23
	30.0	57.5	0.25
	15.5	46.9	0.23
	21.4	50.9	0.28
	22.7	46.7	0.25
	20.9	54.2	0.27
	21.8	53.8	0.18
Mean	22.2	51.9	0.24
SD		3.8	0.03

TABLE IV

PERIPHERAL PLASMA 11-OHCS CONCENTRATION AND HEMATOCRITS IN SIX BEAGLES OVER A 24 HOUR PERIOD

		Time					
		8:42 am	12:31 pm	4:10 pm	8:20 pm	12:05 am	4:03 am
Dog No.	Weight (kg)	µg/ml — Hct (%)	µg/ml — Hct (%)	µg/ml — Hct (%)	µg/ml — Hct (%)	µg/ml — Hct (%)	µg/ml — Hct (%)
D-245	10.9	0.32 — 48.4	0.20 — 48.5	0.29 — 48.7	0.26 — 50.0	0.32 — 49.0	0.25 — 47.1
D-244	10.9	0.29 — 51.7	0.35 — 50.9	0.25 — 44.8	0.25 — 52.7	0.18 — 44.2	0.27 — 49.3
D-251	9.1	0.38 — 58.1	0.35 — 56.3	0.28 — 52.6	0.26 — 57.1	0.25 — 56.8	0.27 — 54.2
D-248	11.8	0.26 — 50.2	0.35 — 53.1	0.23 — 50.5	0.28 — 52.8	0.23 — 52.7	0.25 — 50.0
D-250	15.0	0.36 — 53.3	0.32 — 54.6	0.29 — 47.7	0.21 — 51.5	0.26 — 52.0	0.18 — 50.5
D-249	8.2	0.23 — 50.0	0.23 — 50.0	0.26 — 45.7	-- — 47.8	0.20 — 47.1	0.25 — 49.0
Mean	11.0	0.31	0.30	0.27	0.25	0.24	0.24
SD		0.05	0.06	0.02	0.01	0.04	0.03
SEM		0.02	0.03	0.01	0.01	0.02	0.01
Hct Mean		52.0	52.2	48.3	52.0	50.3	50.0
SD		3.1	2.8	2.7	2.9	4.1	2.3

TABLE V

ADRENAL BLOOD FLOW BEFORE AND AFTER HYPOPHYSECTOMY

(12 Dogs)

Time (minutes)	Adrenal Blood Flow (ml/min/gland)
-20 to -16	2.7, 2.8, 2.8, 2.6, 3.2, 5.4
-11 to -15	----------
-6 to -10	2.3, 2.3, 2.9, 4.4
0 to -5	1.4, 2.1, 2.1, 2.3, 2.6, 3.0
Hypophysectomy	
0 to 5	1.5, 1.5, 1.5, 1.5, 1.8, 1.8, 1.9, 2.1, 2.3, 2.3, 2.5, 2.7, 4.2
6 to 10	0.8, 1.1, 1.6, 1.6, 1.6, 1.8, 1.9, 1.9, 2.0, 2.0, 2.0, 2.5, 2.6, 2.6, 2.8, 2.9
11 to 15	0.75, 1.8, 1.9, 2.6, 2.8, 3.0, 3.2, 3.3, 3.4, 3.5
16 to 20	1.7, 1.9, 2.0, 2.3, 2.6, 3.3, 3.4, 3.7
21 to 25	2.5, 1.8, 2.9, 3.4, 3.6
26 to 30	2.2, 4.1, 2.4, 2.4, 2.5, 2.8, 3.3
31 to 35	2.1, 2.2, 3.4, 3.6
36 to 40	----------
41 to 45	2.9, 2.6, 3.2
46 to 50	----------
51 to 55	----------
56 to 60	1.2, 2.2, 2.4, 2.4, 2.6, 2.7, 3.1

TABLE VI

LIVER BLOOD FLOW BEFORE AND AFTER HYPOPHYSECTOMY

(8 Dogs)

LBF = ml/min/10 kg

Time	LBF	Time	LBF
-20	476	16	501
-17	519	23	550
-15	552	28	470
-11	637	29	738
-10	525, 512	30	592, 520
- 6	529	35	425
- 4	659	39	770, 668
- 3	634, 585	41	646, 529
Hypophysectomy		42	758
2	492	45	570
3	529	49	751
4	544	50	669, 335, 680
5	475	51	533
8	435, 538	56	741
11	445	59	724
15	641, 659	60	738, 592

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