SHORT TITLE FOR BINDING:

METABOLISM OF THE 17-HYDROXYCORTICOSTEROIDS

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SOME STUDIES ON THE METABOLISM OF THE 17-HYDROXYCORTICOSTEROIDS IN THE HUMAN

by

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Thesis

Submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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McGill University,
Montreal.

January, 1961.

ACKNOWLEDGMENTS

The Author should like to express his appreciation to Dr. R. V. Christie, Professor of Experimental Medicine, McGill University, for the opportunity to have done these studies in his department. He is indebted to Dr. J. C. Beck, Royal Victoria Hospital, Montreal, for his immediate supervision, financial assistance, laboratory facilities and general support. Dr. J. S. L. Browne, Professor of Investigative Medicine, McGill University, was very helpful in giving advice, financial support and a technician's salary. He is grateful to Dr. E. H. Venning, Royal Victoria Hospital, Montreal, for having provided laboratory space and many laboratory facilities. Financial assistance from the CIBA Co. Ltd., Montreal, and the National Research Council of Canada were sincerely appreciated. The author held the CIBA Medical Research Fellowship in 1957-58. Acknowledgment of specific technical assistance is made in the appropriate sections of the thesis. Finally, the author would like to say that without the cooperation of the many medical students, nurses, house officers and patients at the Royal Victoria Hospital, who served so willingly as subjects, these

studies could never have been done.

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INTRODUCTION

A. EARLY DEVELOPMENTS

The protean findings on patients with adrenocortical tumors and the fact that the adrenal cortex is requisite to life has long been established. Tilesius, in 1803, described obesity, precocious breast development and hirsutism in a girl of four, found to have a tumor of the adrenal cortex (1). The fatalness of destructive lesions of the "suprarenal capsules" was reported by Addison in 1855 (1). By 1926, Bauman and Kurland had demonstrated a significant decrease in the sodium and chloride contents of the blood associated with a concomitant increase in the potassium and magnesium contents, in cats, following adrenalectomy (2). That same year, using dogs, Rogoff and Stewart confirmed this postoperative fall in blood chloride and went on to report an associated increase in the non-protein, urea and undetermined nitrogen fractions of the blood as well as the development of hemoconcentration (3). Subsequently, they observed that the presence of pregnancy at the time of adrenalectomy would prolong life and, during the same

year, Rogoff and Dominguez described the progressive fall in blood pressure in adrenalectomized male dogs (4, 5). In 1928, Goldzieher reported that the saline precipitate of acid extracts of the adrenal gland were able to prolong the life of adrenalectomized rats (6). He termed the essential substance in the extract "interrenin." Later that same year, Rogoff and Stewart were able to prolong the life of adrenalectomized dogs, first with intravenous Ringer's solution, and, subsequently and more effectively, with various saline and glycerine extracts of adrenal tissue(7, 8). The inferred hormone was termed "interrenalin." Confirmation of these observations came from Hartman et al who employed saline extracts in adrenalectomized cats (9). They suggested the name of "cortin" which won universal acceptance immediately.

In 1928, Swingle showed that nephrectomy would not prevent the post-adrenalectomy decrease in blood sugar and chloride as well as the rise in blood phosphate and sulfate (10). Two years later, he and Pfiffner observed the prolongation of life in adrenalectomized cats treated with lipid solvent extracts of beef adrenals; these extracts were superior in potency to the saline

ones (11, 12). Hartman immediately adopted the use of lipid solvents for adrenal tissue extraction (13). In 1931 Hartman, Brownell and Crosby reported the failure of cortin treated rats to demonstrate a significant drop in colonic temperature following adrenalectomy, as did untreated controls (14). This phenomenon was to find application in a bio-assay for cortin-like activity which was to be employed later in clinical investigation.

By 1931, Perla and Marmorston-Gottesman found that benzene extracts of normal urine contained cortin-like activity (15, 16). Concentrates of their extracts, when administered to adrenalectomized rats on the fifth or sixth post-operative day, prevented death when the animals were challenged with histamine, 200 mg./Kg. Confirmation came from Grollman and Frior within the year (17).

At this point it is necessary to digress momentarily and draw to attention the fact that the early 1930's marked the beginning of the era of adrenocortical enlightenment, a continuing revolution which has seen no end. At first, there was a black magic-like aura about many of the experiments for the few pertinent observations

were not well understood. During this period, the chemists, Kendall and Mason, Reichstein and Wintersteiner and Pfiffner set about to isolate and characterize the hormone or hormones of the adrenal cortex (18, 19, 20, 21, 22, 23). Physiologists in some confusion attempted to further delineate the physiological pathology of adrenalectomized animals under various experimental conditions. Clinicians, anxious to offer a thread of hope to their Addisonian patients, reported successful use of the new lipid solvent extracts of adrenal tissue (24, 25). These extracts were not entirely satisfactory and their potency was not dependable. The drug houses, always anxious to capitalize on any wave of interest, offered an array of adrenocortical preparations, as exotic in name as they were undependable (26). Soon, Loeb was to warn against "the widespread and indiscriminate administration of cortical extract in small and infrequent doses to all those who are 'weary and heavy laden'" (27). Also it was the time of the great furor over whether Cushing's syndrome was due to a pituitary or adrenocortical lesion. The argument has not been entirely settled yet (28, 29, 30, 31). All of this is outside the scope of this review in which

the primary object is to recall the development and application of effective laboratory methods for the assessment of adrenocortical activity in patients. It is important, however, to remember that these developments occurred in this particular context.

B. THE BIOLOGICAL ASSAYS

The various bio-assays employed in the determination of the cortin activity of body fluids were developments of the methods originally used to standardize adrenocortical extracts. It is appropriate, therefore, to review those methods at this point.

By far the most popular laboratory animal was the rat but dogs, cats, mice and even fish were used. As to the rats, following adrenalectomy, the test extracts were administered; certain of the animals were given reference (standard) extracts of known potency while others were withheld from any treatment and used as controls. Potency was correlated with the ability to significantly alter various parameters: survival, growth or resistance to stressful challenges. Hartman and Thorn described a growth and survival assay in 1930 (32). Subsequently, in 1936, Schultzer as well as Cartland and

Kuizenga developed rat survival tests (33, 34). The latter, using 50 - 60 gram rats, administered daily subcutaneous injections of the test extracts. One unit of activity was equivalent to the smallest daily dose sufficient to maintain 80 percent of the treated rats for 20 days and to permit a 20 gram weight gain. In 1943, Kuizenga et al found that this daily dose was equivalent to between 1/8th to 1 mg. of 17-hydroxy-corticosterone (35). Reichstein and Kendall had isolated and described this compound as well as 17-hydroxy-11-dehydrocorticosterone by 1937 (36, 37). A growth curve with the log of the dose plotted against the weight gain was used by Grollman to determine potency (38). Perla and Marmorston-Gottesman assayed the ability of the test rats to resist histamine and typhoid vaccine (15, 39). A unit of activity was defined as the minimal amount of cortical extract, administered on the fifth or sixth post-operative day, which was able to prevent death when the animal was challenged with histamine, 200 mg./Kg.

Widstrom, in 1935, using albino rats and albino mice observed a stepwise decrease in colonic temperature following adrenalectomy; this was not observed in the

intact controls (40). This confirmed the earlier observations of Hartman's group (13). He found that the ability of a test or standard extract to inhibit this reaction was directly proportional to its cortin-like activity. Horvath showed that the temperature did not fall following unilateral adrenalectomy (41, 42). Subsequently the "cold test" of Selye and Schenker was developed (43). This involved a determination of the ability of adrenalectomized rats, weighing 35 to 50 grams, to withstand exposure to temperatures ranging from 2°C. to 5°C. The "cold unit" was defined as the minimal amount of extract necessary to maintain 2/3rds of the treated rats when 2/3rds of the controls had died. One cold unit was later observed to be equivalent to the activity of 1 mg. of crystalline corticosterone (44).

A fatigue test was described by Everse and de Fremery (45). The gastrocnemius muscle of an adrenalectomized rat was stimulated tetanically and the contractions recorded. One fatigue unit was defined as the minimal daily dose, administered in two injections, which could maintain normal contractile power. Ingle et al introduced modifications to this assay; they attached a

100 gram weight to the leg and used faradic stimulation, at a frequency of 3 stimuli per second (46). Eventually, 1 fatigue unit was found to be equivalent to the biological activity of 0.2 mg. of 17-hydroxy-11-dehydrocorticosterone (47).

Gaarenstroon, Waterman and Laqueur used a "swimming test" (48). Normal rats were said to be able to keep the tips of their nose above water for 30 to 45 minutes. Adrenalectomized rats could not do this unless pretreated with adequate amounts of adrenocortical extract. Their swimming time was proportional to the potency of extracts.

Harrop, Swingle and Pfiffner developed a dog assay in which 1 dog unit was equal to the minimal daily dose per kilogram of body weight required to maintain the non-protein nitrogen of the blood within normal limits over a test period of 7 to 10 days; later they used the blood urea nitrogen (49, 19). Uyldert correlated temperature drops and decreased food intake in his dogs with the potency of the test extracts (50).

Hartman and Pohle employing adrenalectomized cats followed the daily food intake (51). The dosage of

the test extracts was halved every fourth day and the appetite carefully followed; the minimal daily dose, divided into two daily injections, which was capable of maintaining a normal diet was determined.

In 1939, Schacter and Bebee described a guinea pig survival assay (52). It was their opinion that the guinea pig was easier to operate upon, seldom had accessory adrenal tissue, required smaller doses of extract than did the cat or dog and had a more uniform survival period than the rat. Previously, Zwemer and Jungeblut, Wolfrom and Zwemer and Herbrand had shown that adrenocortical extracts could protect guinea pigs against diphtheria toxin and anaphylactic shock (53, 54, 55). The guinea pig has never become a popular animal among adrenocortical investigators.

A unique assay was reported from France by Santa and Veil and Giroud, Santa and Martinet (56, 57). They found that adrenocortical extracts and desoxycorticosterone caused melanphore contraction in the Cyprimus carpio (carp) and they developed this into a seemingly quite sensitive assay. However, Smith and Hartman later showed that the effect was not specific for adrenocortical

extracts (58).

It had been noted by Bennett in 1937 that the liver of intact rats, treated with adrenocorticotropic hormone had a higher glycogen content than that of untreated controls (59). Their blood sugar levels were correspondingly elevated. Two years later, Long, as well as Corey and Britton, observed the same phenomena in rats treated with adrenocortical extract (60, 61). Grattan and Jensen, describing similar findings in mice, noted that corticosterone, 17-hydroxycorticosterone and 17-hydroxy-11-desoxycorticosterone exerted such an "anti-insulin" effect but that desoxycorticosterone and desoxycorticosterone acetate showed little of this activity (62, 63). Reinecke and Kendall incorporated these observations into a dependable biologic-chemical assay with the measurement of a final chemical reaction correlated to the cortin potency (64). Adrenalectomized rats, fasted and weighing 140 - 150 grams, were tested on the fourth post-operative day. Serial, hourly injections were used and immediately upon death the livers were removed and dropped into hot 30 percent potassium hydroxide. Thereafter, the glycogen content was determined by the method

of Good, Kramer and Somogyi (65). The glycogenic unit was defined as equivalent to the biological activity of 1 microgram of 17-hydroxy-11-dehydrocorticosterone. Confirmation of the accuracy of the method was quick to come from Bergman and Klein, as well as Venning, Hoffman and Browne (66, 44). Venning, Kazmin and Bell soon adapted this assay to adrenalectomized mice and noted that the sensitivity was increased if a glucose load was administered to the animals just prior to the test period (67).

During this period two other biologically active factors, which could be shown to be definitely separable from cortin during special extraction of adrenal tissue were reported. These were the sodium retaining factor of Thorn and the adrenocortical androgenic compounds (68, 69, 70, 71, 72). It is not within the scope of this discussion to comment upon the isolation, identification and methods of assay for these factors.

There were many disadvantages to the above mentioned bio-assays. Within one laboratory or between any two laboratories there were innumerable points of potential variability. Hartman and Brownell pointed out that, if results were to be meaningful, it was necessary,

while using albino rats, to have them of uniform stock, fed a standard diet, exposed uniformly to a minimum of anesthesia and operative trauma and kept at 27° to 28°C. throughout the test period (73). Cleghorn found that the survival period of the untreated control rats varied directly as the weight/age ratio (74). Bliss and Cattell found the slope of the dose regression lines of standards and unknowns was the same but Olson, Thayer and Kopp could not agree (75, 76). The latter authors also found that the minimal daily dose of desoxycorticosterone required for rat growth and survival was 100 µg. which was 1/10th of the 1 mg. dose reported by Grollman (76, 77). D'Armour reported that slightly less than 3 percent of the test rats survived adrenalectomy indefinitely (78). He found that the best time for the test period was immediately following adrenalectomy. As to the cold test, Vogt found that intact rats in some strains could not survive the cold (79). Dorfman, employing the same test, demonstrated the differing degrees of biological activity in the various compounds used as standards (80).

Attempts were made to compare the assessment of biological activity in different laboratory animals.

Cleghorn found cats to require ten times the amount of adrenocortical extract per kilogram of body weight than did dogs (81). Likewise, Cartland and Kuizenga reported that one rat unit (survival test) was equivalent to 22 dog units (34). Venning et al found reasonable agreement upon comparison of the cold, survival and glycogen deposition tests in the rat (44). Olson et al, however, were unable to correlate glycogen deposition and survival time in the rat; also in response to the same dose of extract they observed variations in growth (82). Browne, comparing the glycogen deposition assays as done in three laboratories, noted the following points of variability: 1) different methods of collecting the urine, 2) differences in the extraction of urine (variation in solvents, amounts and pH), 3) variations in the methods of purifying the extracts, 4) variation in the stability of extracts, 5) different methods of preparing the extracts for administration to the animals, 6) differences in the animals (variation in stock, age and weight), 7) variability of surgical techniques during adrenalectomy, 8) variations in the post-operative treatment of the rats and 9) differences in the protocols and criteria of potency

(variation in the number of injections, interval and standards) (83).

Obviously, these assays were involved, complicated, expensive, time consuming and contained potentially large errors. It was particularly important to be definite about methods, for each group varied as to what they started with, processed and ended up with. Yet, these methods were all that was available at the time and despite the hazards, much significant data was obtained through their clinical application.

A few years after the observations of Perla and Marmorston-Gottesman on normal urine, Anderson and Haymaker, employing the rat survival assay, demonstrated cortin-like activity in the extracts of blood and urine from patients with Cushing's syndrome (84, 85, 86). Extracts from the blood and urine of normals could not be shown to possess such activity.

Browne and Weil concluded from observations by Picon and from Selye's hypothesis of the adaptation syndrome that cortin-like activity should be high enough to detect in the urine during acute stress; in 1940, using the cold test they observed such activity in the urine

of a patient acutely ill with influenza (87, 88, 89). Subsequently, they demonstrated a similar degree of activity in urine during the surgical stresses of appendectomy, dilitation and curettage, gastric resection and colostomy (90). Similar observations, by them as well as by Albright, were made on urine from patients with the adrenogenital syndrome, hypertension, subacute bacterial endocarditis, chronic and acute infections including pneumonia, Cushing's syndrome and those treated with desoxycorticosterone (91, 92).

Dorfman confirmed the presence of cortin-like material in urinary extracts from patients who were similarly stressed (93). Vogt employed the cold test to demonstrate the presence of increased amounts of cortin-like material in the venous effluent of dogs' adrenal glands (79). She estimated that the daily output of cortin from the adrenals was equivalent in activity to the extract of 0.6 grams of adrenocortical tissue per kilogram of body weight.

By means of their melanphore contraction assay, Giroud, Martinet and Pacoret showed that extracts of normal urine contained 40 to 50 units of activity per 24

hours, urine of a patient with Cushing's syndrome contained 100 units but that of an Addisonian patient had 15 units (94).

Following the development of an extraction method to increase the cortin-like content of their urinary extracts, Venning, Hoffman and Browne turned to the glycogen deposition assay of Reineche and Kendall and were able to demonstrate cortin activity in normal urine; in the urine of patients undergoing surgical stress they found the activity was 3 to 30 times the amount in normal urine (95, 44). Then they adapted the method to adrenalectomized mice and showed that the level of urinary cortin activity in their patients could be correlated with the degree of negative nitrogen balance which they developed during acute stress; they could not quantitatively relate these changes to fluctuations in the levels of the urinary 17-ketosteroids (96, 97, 98). This group went on to show that the elevated urinary corticoids of acute stress were not altered by variations in the protein content of the diet, that these levels could be elevated in active acromegaly, that testosterone proprionate therapy could diminish the level and that a rather crude

preparation of ACTH (60 to 100 mg., intramuscularly) could not be shown to alter the level (99, 100, 101). Albright, Reifenstein and Forbes noticed that the fluctuation in the urinary corticoids could be related inversely to the lymphocyte count (102). Suspecting that adrenocortical hyperfunction was physiologically present during pregnancy, Venning followed the urinary corticoid excretion in several women throughout pregnancy and found that there was a progressive increase during the second and third trimesters (103).

Recalling that Cohen and Marrian and Dobriner et al had shown that urinary androgens are excreted as complexes with glucuronic acid, Dobriner found that he could increase the yield of cortin-like material from the urine by continuous extraction at pH1 for 48 hours (104, 105, 106). He concluded, therefore, that the cortin-like material was also excreted in a conjugated form and that the yield could be augmented by acid hydrolysis.

Early in this period, Browne and Weil had shown that extracts from patients given desoxycorticosterone contained increased amounts of cortin activity and Vogt, in the dog, traced this activity to its source, the

adrenal gland (91, 76). Despite the isolation and description of many adrenocortical compounds during these years none were so identified in the urine, although Butler and Marrian had isolated certain Δ^4-pregnene derivatives from the urine of patients with the adrenogenital syndrome (106, 107, 108). Dorfman et al, using the cold test, showed that cortin-like material appeared in the urine of adrenalectomized monkeys, adrenalectomized and castrated monkeys and in Addisonian humans only when they were treated with adrenocortical extracts (109, 110). Subsequently, Hoffman demonstrated that cortin-like material, which had been isolated from pooled human urine, had a melting point range (uncovered) of 234° to 236°C. and could reduce alkaline silver (111).

During the period of the bio-assays, various investigators found that adrenocortical extracts could protect laboratory animals against severe and otherwise fatal surgical trauma (88, 112, 113). Ivory felt that by its use he had saved from death three cases severely burned in the Hindenberg Disaster of 1937 (114). Perla and Marmorston observed that these extracts could show a favourable effect on the clinical course of patients ill

with pneumonia, malaria or grippe (115).

C. CHEMICAL METHODS

Following the development by Zimmerman, 1935, of a reasonably reliable colorometric method for the determination of the androgenic content of biologic fluids, it was felt that a similar technique could be developed for the corticosteroids (116). Although it bears emphasizing that chemical activity cannot be directly correlated with biologic activity, Fieser, Fields and Lieberman reported two such chemical methods: 1) the establishment of a glycol cleavage at the oxygen atom on carbon-17 with periodic acid or lead tetra-acetate and 2) the conversion of a 1, 2 or 1, 3 glycol to an alcohol extractable acetal derivative (117). These methods, however, did not receive wide acceptance. By 1946, Heard and Sobel had reported that corticosteroid extracts, like certain sugars, possessed the ability to convert phospho-molybdic acid reagent (Folin-Wu) to molybdenum blue (118, 119). Venning found that there was a reasonable correlation of this activity with the glycogen deposition activity (83, 120). Concomitantly, Talbot et al developed a colorometric method dependent upon the copper reducing

capacity of the ketonic fraction of corticosteroid extracts following hydrolysis with Gerard's reagent (121).

Lowenstein, Corcoran and Page first reported the subsequently popular formaldehydogenic method in 1946 (122, 123). Upon oxidation of the corticosteroids with periodic acid, formaldehyde is formed, one molecule for each molecule of oxidized steroid. The formaldehyde, following distillation, was determined according to the method of MacFadyn (124). Daughaday and workers adopted the method and, following a few modifications, found it dependable (125, 126). They did note that the results were not easily correlated to those of the glycogen deposition assay because the method also measured the nonglycogenic steroids with 21 carbon atoms. This method enjoyed wide popularity between 1948 and 1954. Some investigators had difficulty in reproducing their results; recoveries were variable and the formaldehyde did not always distill completely. Therefore, diffusion methods were tried and termed successful (127, 128, 129). Later, Hadd and Perloff developed a special distillation apparatus and reported recoveries in the neighborhood of 98% (130).

In 1940, Bennet observed that steroid containing tissue and phenylhydrazine reacted to produce yellow coloured hydrazones (131). Ashbel and Seligman incorporated this into a histochemical, analytic method for the adrenal cortex in 1949 (132). One year later, Porter and Silber reported its further application as a dependable colorometric assay of the corticosteroid activity of adrenocortical compounds and extracts (133). Extraction was done with chloroform and the extract was divided so that the sulfuric-acid-phenylhydrazine chromagens were determined on one half and the sulfuric acid chromagens on the other half. Since phenylhydrazine in sulfuric acid absorbs light at 410 mμ and certain corticosteroids develop colors in sulfuric acid more concentrated than 1.8:1, they advised using phenylhydrazine and sulfuric acid in somewhat less than optimal concentrations. Their reagent mixture consisted of 65 mg. of phenylhydrazine hydrochloride in 100 mls. of dilute 1.63:1 sulfuric acid. Following evaporation of the extracts, 8 mls. of this reagent was added to the dry residue in a test tube. For the blanks of both samples and standards the phenylhydrazine was omitted. All tubes were held in a water

bath at 60°C. (± 1 degree) for twenty minutes. The absorption at 410 mμ. in a spectrophotometer, when read against a sulfuric-acid-phenylhydrazine reagent blank, was determined. The true optical density of the phenylhydrazine chromagens was determined by subtracting that of the sulfuric acid chromagens from that of the sulfuric-acid-phenylhydrazine chromagens. This method they found satisfactory for the determination of the 17-hydroxy-corticosteroid content of steroid standards, adrenal tissue extracts and extracts of blood and urine to which 17-hydroxy-11-dehydrocorticosterone had been added. However, they could not find any demonstrable phenylhydrazine chromagens in the blood or urine of a dog given large doses of 17-hydroxy-11-dehydrocorticosterone.

The following year, employing this method, Carroll, McAlpine and Noble found phenylhydrazine chromagens in normal urine which had been extracted at pH2 (134). The range for both males and females, per 24 hours, was equivalent in chromagen content to 0.5 to 1 mg. of 17-hydroxy-11-dehydrocorticosterone (cortisone). Increased levels were found in the urine of patients receiving ACTH, cortisone or under the stress of gastrectomy. It remained

for Nelson and Samuels to introduce modifications which increased the sensitivity of the method so that the corticosteroid content of a small amount of plasma could be determined (135, 136). They purified the extracts by employing Florisil column partition chromatography, reducing the volume of the reagents (.2 mls. methanol and 3 mls. of sulfuric-acid-phenylhydrazine reagent), using microcuvettes and determining the optical density at 370, 410 and 450 mμ. so that the Allen correction could be used and the division of the sample avoided (137).

Subsequently, numerous modifications for extraction and purification of phenylhydrazine chromagens were introduced, especially as to the determination of the plasma 17-hydroxycorticosteroids; this is probably due to the fact that plasma, because of the presence of many interfering substances, is more difficult to handle than urine. Butanol extraction at pH1 was introduced by Reddy for both urine and plasma (138, 139, 140). Sweat and Bayliss developed a modification of the Nelson-Samuels method and Bliss et al found the results by this modification comparable to the original Nelson-Samuels method (141, 142, 143, 144). Eik-Nes, Nelson and Samuels

pointed out, after many investigators had expressed difficulty in employing their method, that it was important to use freshly distilled chloroform and Harwood recommended double distillation of chloroform over calcium carbonate as well as careful cleaning and heat activation of the Florisil (145, 146). Bondy and Altrock and Kassenaar and workers employed multiple liquid/liquid partitions for purification (147, 148, 149). Girard's reagent was used for purification by Coste and Delbarre as well as Vestergaard (150, 151). Silber and Porter published a modification of their reagent in 1954 in which ethanol and dilute sulfuric acid, 1.63:1, were combined, 1 volume: 2 volumes, for micro determinations (152). Silber and Busch as well as Peterson and his coworkers showed that methylene dichloride was a preferable solvent for extraction, because it could be stored for longer periods following distillation without significant decomposition and its partition coefficient for the 17-hydroxycorticosteroids was greater than that of chloroform (153, 154).

There are probably many other modifications, published and unpublished, for the determination of

phenylhydrazine chromagens. Gold and Ward et al have compared the published data of many (155, 156). Although most investigators claim recoveries in the neighborhood of 85% to 105%, there is a large variation as to their normal means. For the means of the plasma values reported by various workers the range is from 5.9 to 20.5 µg./100 mls. of plasma. The reason for this is probably due to the variation in the normal material i.e. variation as to numbers, ages, sex, methods for the collection of samples, storage and pooling of samples. Nevertheless variations in the methods also exist. The determination of the phenylhydrazine chromagens has had wide application but, as in the case of the bio-assays, it is important to be definite as to what is used for the sample, how it is processed and what is finally measured. Furthermore, the comparative convenience of the chemical methods should not obscure the fact that with such methods we are measuring chemical activity which may not be consistently correlatable to biological activity.

Chemical methods other than those in which phenylhydrazine chromagens are determined have been developed; they, however, have not been used to as great

an extent. Mader and Buck have described the blue-tetrazolium reaction for the measurement of all steroid compounds with an alpha ketol side chain on the carbon-17 (157). Weichselbaum and Margraf have favourably compared its results with those of the Porter-Silber method whereas Marks et al were not able to do this (158, 159). Norymberski reported a method which has enjoyed wide popularity in Europe (160). This method determines the ketogenic steroids (those with the configurations of a 17, 21-diol-20-one;17,20,21-triol; and 17, 20-glycol) by converting them to ketosteroids with sodium bismuthate and subsequently performing the Zimmerman reaction. Gornall and MacDonald have found that by using 2, 4-dinitrophenylhydrazine they can determine, at different stages in the color development, those compounds with a Δ^4-3-ketone configuration and those with only a 20-ketone configuration (161). Furthermore, because of differences in maximum absorption, they can also differentiate between those steroids containing a 20-ketone grouping with a 17-hydroxyl radical and those without. Isonicotinic acid hydrazide has been shown by Umberger to form hydrazones with steroids which contain a Δ^4-3-ketone configuration (162).

Weichselbaum et al have employed this method and feel that of the various chemical methods this one most closely approximates the determination of the biologically active corticosteroids (163). Sweat has reported a fluorometric method for measuring the corticosteroids and Morris has described a polarographic (measurement of electrical conductance) method (164, 165).

During this period further investigation of the methods of hydrolysis of the conjugates was undertaken. Following the observations of Cohen, Marrian and Odell, in 1936, who introduced the acid hydrolysis of urinary glucuronides of estriol, and Buehler, Katzman, Doisey and Doisey, in 1949, who used bacteria to hydrolyze other conjugated steroids, Venning and Cohen made preliminary reports in 1951 as to the use of β-glucuronidase in corticosteroid hydrolyses (104, 166, 167, 168, 169). Glenn and Nelson confirmed these findings and reported that the 17-hydroxycorticosteroids, as determined following hydrochloric acid hydrolysis and enzyme hydrolysis, were of comparable levels (170). This was not found to be so for the 17-ketosteroids. Bongiovanni reported a method for the determination of 17-hydroxycorticosteroid glucuronide

levels in the blood using enzyme hydrolysis (171). Klein et al determined the level of conjugates in the plasma by hydrochloric acid hydrolysis (172).

With the application of the above described chemical methods, there were significant gains in our knowledge of the activity of the corticosteroids. Employing the phosphomolybdic acid method Heard, Sobel and Venning observed increased excretion levels in the dog and human while on ACTH (173, 174). In Addisonians, urinary levels were 1/3rd those of normal and those of Cushing's syndrome were 2 to 5 times normal. Patients given dehydrocorticosterone and desoxycorticosterone showed no increase (120). Burns et al, using this method, found an increase in the urinary level following the intravenous administration of ACTH to a human (175).

Albright reported that the urinary copper reducing corticosteroids assayed according to the method of Talbot were increased in the human following the administration of ACTH (176, 101). Reifenstein reported that this fraction was also increased in normal subjects while on a high protein diet but that this was not the case with Addisonians on a similar diet (102). Venning

had observed no change in urinary glycogen deposition activity despite variations in the protein intake (102).

By 1947, various investigators were turning to the formaldehydogenic method; Mason reported observing an increase in formaldehydogenic corticosteroids in the urine following the administration of ACTH (177, 178). This was confirmed by Mc Intosh, Singer and Hoffman and later Beck (179, 180). Tobian found that the urinary formaldehydogenic fraction was elevated in late pregnancy and even higher in a case of toxemia (181). Chart, Shipley and Gordon could not confirm this but they did find an increase in the sodium retaining activity of urine of patients with toxemia (182). Previously, Venning had found that the glycogen deposition activity of urine in cases of toxemia was decreased but in 1952 she reported that the formaldehydogenic corticosteroids and the mineralocorticoids were elevated during toxemia (183). Lloyd et al reported that they too found urinary formaldehydogenic activity high during toxemia (184). Wolfe and Paschkis employed this assay to study the urine of diabetics and found that half of these patients had reduced levels (185). They did not attempt a correlation with the degree of

glycosuria, however; furthermore, glucose itself is formaldehydogenic if not eliminated. Romanoff, Plager and Pincus studied the diurnal variation in the excretion of both the formaldehydogenic and the phosphomolybdic acid reducing fractions (186). They found the highest excretion rates in the morning.

Thorn et al, in 1952, reported the first ACTH adrenocortical stimulation test in which the measurement of urinary phenylhydrazine chromagens was undertaken (187). ACTH was administered intravenously over a 2-hour period; patients with adequate adrenocortical reserve were said to show an increase in the urinary 17-hydroxycorticosteroid excretion during the two hours following the cessation of the ACTH. In the subsequent year, the same group reported a more satisfactory test with 15 - 20 I.U. of ACTH given intravenously over an 8-hour period each day for 2 or 3 days (188). The urinary excretion of the 17-hydroxycorticosteroids was followed throughout a control period and the treatment days. This was necessary in order to distinguish the primary adrenocortical atrophy of Addison's disease from the secondary atrophy found in hypopituitary states and hypothyroid states. Eik-Nes et al preferred

a 6-hour test with ACTH, 25 I.U. administered intravenously over the entire period and the plasma 17-hydroxycorticosteroids determined at 0, 2, 4, and 6 hours (189, 190). Fifty units of ACTH over the same period of time produced no greater response. Liddle et al reported that patients with Cushing's syndrome had a response to ACTH, as determined by the urinary 17-hydroxycorticosteroid excretion, of greater than 50% over the level of the control period; normals were less than 50% (191). Laidlaw and co-workers were in agreement (192). Christy, Wallace and Jailer observed a greater than normal responsiveness in the plasma 17-hydroxycorticosteroids in patients with Cushing's syndrome (193, 194). Confirming this Lindsay, Migeon and Nugent also observed a greater than normal rise in these chromagens in the urine (195). Soffer noticed, as judged by the response of the plasma 17-hydroxycorticosteroids, only 2/3rds of his patients with Cushing's syndrome were hyperresponders (196). Two with adrenocortical tumors did not respond. Sayers et al and Haydar et al called attention to the observation that certain Addisonians had low but detectable levels of plasma 17-hydroxycorticosteroids which failed to increase follow-

ing ACTH (197, 198). Bliss and co-workers showed that these plasma chromagens could also be elevated by the administration of bacterial pyrogens, insulin, toxic doses of histamine and the precipitation of alcohol-antabuse reactions (199). The implication was that such simulated stress caused the release of ACTH.

As to the use of ACTH in these tests, Bayliss and Steinbeck noted with 20 I.U. given intramuscularly, intramuscularly in gel and intravenously over a 6-hour period that the duration of response was 3, 5 and 10 hours respectively (200). Forsham noted that a certain amount of the older and relatively impure preparations of ACTH were inactivated if given intramuscularly; this was not true of the newer preparations (149). Eik-Nes et al have observed that the responses of plasma 17-hydroxycorticosteroids to various commercial preparations of ACTH, employing the same dose intravenously, are comparable (201).

In 1954, Tyler et al demonstrated that there was a diurnal variation in the 17-hydroxycorticosteroid level of the plasma such that the highest values were to be found between 6 and 8 a.m. (202). Laidlaw et al

observed a similar pattern of variation in the urinary excretion rates of the same chromagens (203). Migeon observed a similar variation in the plasma of blind subjects and night workers (204). Later Brown and co-workers observed a diurnal variation in the level of the conjugates of the 17-hydroxycorticosteroids in the plasma; it followed that of the free hormone by about two hours (205). Forsham suggested that such variation was due to changes in the degree of responsiveness of the adrenal cortex to ACTH (149). It remained for Nugent and others to show that a dose of ACTH as low as 0.8 I.U. administered intravenously over 24 hours could destroy such a diurnal pattern and that the adrenal cortex was equally responsive to the same dose of ACTH regardless of the time of day or night (206, 207). They concluded that such a diurnal variation was a reflection of variations in the levels of ACTH.

The group in Salt Lake City, in 1953, under the leadership of Samuels and Tyler studied the rate of disappearance of the 17-hydroxycorticosteroids from the plasma following an intravenous loading of "cortisol" (17-hydroxycorticosterone), 1 mg./Kg. of body weight, which was administered over a 30-minute period, employing

the Nelson-Samuels micro method (208, 209, 149). The so-called biologic half life was found to be in the neighborhood of 2 hours. The rate of disappearance was unaltered with epinephrine administration. It was reported to be prolonged during surgical stress, in liver disease and in advanced renal disease (210, 211). The tetrahydrocortisol disappearance, however, was not prolonged in liver disease. This led to the conclusion that the rate of reduction (i.e. the rate of metabolizing cortisol to tetrahydrocortisol) but not the rate of conjugation was impaired by liver disease. The BSP removal test was reported to be temporarily impaired during surgical stress in some of their cases (212). Klein, Papadatos and Fortunato confirmed these findings in patients with liver disease (172). Szenas and Pattee reported an increased disappearance rate in the obese using similar techniques (213). Moore found an increased responsiveness to ACTH during the stress of surgery as determined by the changes in the plasma 17-hydroxycorticosteroid levels (214). Gemzell, in 1953, reported an elevation of the plasma 17-hydroxycorticosteroids during the last half of pregnancy (215). Others have confirmed this

and delineated a steady increase during the second and third trimester to levels of 20 to 40 µg./100 mls. (216, 217, 218). Garst and Assoli and Christy, Wallace, Gordon and Jailer reported that the cortisol disappearance was delayed in late pregnancy (219, 220). The tetrahydrocortisone disappearance was correspondingly delayed for unexplained reasons. Allied to these observations are those of Maengyn-Davis and Weiner, who have noted that the urinary 17-hydroxycorticosteroids are elevated in the 2nd and 3rd week of a normal menstrual cycle, and those of Wallace and Carter, who found that plasma 17-hydroxycorticosteroids are elevated and their rate of urinary excretion suppressed during ethinyl estradiol therapy (221, 222). It is also of note that Gemzell and Notter have shown that androgen administration can prevent the increase of plasma and urinary 17-hydroxycorticosteroids in normal subjects during surgical stress and decrease these levels in adrenalectomized patients, on cortisone replacement therapy, to the point of inducing an Addisonian crisis (223).

The adrenal tissue secretion rate was calculated by Bondy and Altrock (224). They catheterized the left

renal vein and determined the venous-arterial (right arm) difference in the plasma 17-hydroxycorticosteroids. This was multiplied by the plasma flow to give a daily excretion rate of 15 to 25 mg. Silber, calculating from the total (free and conjugated) phenylhydrazine chromagen content of human urine, estimated 30 to 60 mg. per 24 hours (225). Moxham and Nabarro correlated oral cortisol dosage with the urinary excretion of the 17-hydroxycorticosteroids and the ketogenic steroids; they estimated the daily production in terms of the former as 9.5 to 45 mg. daily and of the latter as 5.5 to 49 mg. daily (226).

Application of the colorometric methods to the study of blood transport and protein binding of the 17-hydroxycorticosteroids has been limited. Eik-Nes reported that human albumin had an affinity for the 17-hydroxycorticosteroids but that a "specific mucoprotein," not further identified, had a markedly higher binding capacity (227). The conjugates were reported to be bound to a lesser degree and he concluded that binding was inversely proportional to the number of polar groups contained in the molecule. Daughaday performed equilibrium dialysis and found that an average of 94% of the free and 65% of

the glucuronides of the 17-hydroxycorticosteroids were non-dialysable (228). In an in vitro study, Westphal found that human albumin, as opposed to milk, bovine albumin and gelatin, had a high extinction coefficient for cortisol and desoxycorticosterone (229). He employed ultraviolet absorption techniques.

A few studies as to the renal handling of these hormones have been undertaken employing these chemical methods. In 1955, Weichselbaum and Mendeloff reported that the renal clearances of the free 17-hydroxycorticosteroids was 2.5 to 5.0 mls. of plasma per minute while that of the conjugates was 54 to 74 mls. of plasma per minute; they did not report as to their methods and techniques (230). Bongiovanni and Eberlein gave large oral doses of tetrahydrocortisol (THF) to a normal individual and followed the blood levels and the urinary levels during several 20-minute clearance periods (231). They observed that the average clearance values for the free 17-hydroxycorticosteroids during these periods was 14.9 mls./min. and that of the glucuronides was 44.5 mls./min. They used the totally extractable chromagens of the plasma to determine the plasma values which were filtered at the

glomerulus. They did not take into account the effect of protein binding. Daughaday estimated renal clearances before and after oral cortisone in several normal individuals employing a similar technique; his clearance periods were 2 hours in length (228). He found the clearance of the free fraction (phenylhydrazine chromagens) ranged from 3.5 to 12.0 mls./min. and that of the glucuronides from 51 to 192 mls./min. Some of the glucuronide clearances were greater than the endogenous creatinine clearance and he concluded that there could be tubular excretion of the glucuronides; he assumed that the free clearances were low because of the effect of protein binding. Although he did perform equilibrium dialysis on the plasma specimens, he used the total (dialysable plus nondialysable) free and glucuronide plasma values for his calculations (232). He also noted that the clearances of the glucuronide values were slightly reduced if the subject was given probenecid. In similar studies, Kornel and Wroclaw confirmed these observations in normals; they did, however, find that the clearance of the conjugated fraction was about 50% of normal in a series of hypertensive patients (233).

During this period the determination of phenylhydrazine chromagens became a routine procedure. Many significant observations other than those mentioned above have been published. It is pertinent here to mention only those reports which bear directly upon our understanding of the metabolism of the 17-hydroxycorticosteroids.

It was during this period that the main compounds which are assayed as phenylhydrazine chromagens were isolated and characterized. Mason employing the classical analytic methods of organic chemistry isolated 17-hydroxycorticosterone (cortisol or compound F) from urine in 1948; the following year, he isolated 17-hydroxy-11-dehydrocorticosterone (cortisone or compound E) from the urine of an Addisonian receiving cortisone (234, 235). It was in 1951 that Zaffaroni described his method of paper chromatographic separation of adrenocortical hormones (236). He and others isolated 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone from human urine (236). Burton and others were able to isolate THE (tetrahydrocortisone) and THF (tetrahydrocortisol) following β-glucuronidase incubation of human urine (237). Gray reported that a study of the density of the various

hormone spots on the paper chromatograms (Bush system) revealed that of the THE to be 10 to 30 times more dense than those of 17-hydroxy-11-dehydrocorticosterone (compound E) and 17-hydroxycorticosterone (compound F) which were of the same degree of density (238). Schneider calculated from his chromatograms that human male urine contained 51 µg./L. of compound E and 214 µg./L. of THF (239). De Courcy and co-workers found that neutral chloroform extracts of urine contained compounds E and F while large amounts of THF were extractable following beta-glucuronidase incubation; insignificant amounts of chromagenic material were extractable following hydrochloric acid hydrolysis at pH 1 (240).

Nelson and Samuels, using the Zaffaroni system, showed that the 17-hydroxycorticosteroids in human plasma, as determined by their method, was essentially compound F (135). Migeon et al confirmed this using a Bush system (241). Applying plasma extracts to the Zaffaroni system Bongiovanni was able to isolate compounds E and F before hydrolysis; after beta-glucuronidase hydrolysis, he isolated THE and THF (231, 242). Morris employed his polarographic method following partition chromatography

and found normal plasma contained the following concentrations: 3.6 µg./100 mls. of compound A (dehydrocorticosterone), 9.1 µg./100 mls. of compound B (corticosterone), 4.6 µg./100 mls. of compound E, and 9.8 µg./100 mls. of compound F (165). These values were increased in plasma during late pregnancy and ACTH therapy; however, the degree of increase for the various compounds was subject to large individual variation.

D. CURRENT INVESTIGATION

Much useful information was made available through the use of the chemical assays but due to their inherent limitations certain problems remained to be approached by other and newer methods. Within the past 5 years, following the development of methods employing radioisotope dilution techniques, plasma electrophoretic radioautography, plasma ultra-filtration and more highly refined paper chromatographic systems, certain of these problems have been tackled.

Applying the analytic method described by Rittenberg and Foster to the use of 4-C^{14} labelled cortisol administered to patients along with a carrier of reasonably large dosage, Peterson found the biological

half life to be 115 to 120 minutes (243, 244, 245). He found that over 90% of the administered radioactivity appeared in the urine within 72 hours and that this was almost all incorporated into the conjugated fractions. Only traces of radioactivity appeared in the feces and 4% was recovered from the bile of a patient with an indwelling T-tube. Shortly, Sandberg and co-workers and Sheurer and Bondy confirmed the suitability of the method and reported similar disappearance rates using tracer with carrier (246, 247). Migeon et al observed a shorter biological half life (80 minutes) when tracer was administered alone (248). Samuels and others calculating from the plasma 17-hydroxycorticosteroid level and the diminishing radioactivity in the plasma estimated the daily cortisol production of the adrenal glands to be 37 mg. (249). By interpolating the linear curve of the cortisol disappearance studies, in which only tracer has been used, back to zero time an "apparent volume of distribution" would seem to approximate the extracellular fluid volume (250). This cannot be accurately done with large doses of exogenous hormone or tracer and carrier together because of the development of unphysiologic concentrations (247). Such

calculations are based on the assumption that the hormone is equally distributed throughout such volume.

Employing such methods, it has been shown that the disappearance rate of cortisol is prolonged in patients with liver disease, although they show a normal rate of disappearance for cortisone and THF (250, 251). The biological half life of infused cortisol has been found to be about twice normal in late pregnancy and in the newborn (252, 253). Nugent and workers have demonstrated that the rate of cortisol disappearance is constant throughout the 24 hours of a day (205).

More recently, turnover rates have been studied by employing one of two techniques. If the declining specific activity in the plasma is plotted against time, the negative slope of the line is a measure of the rate of turnover (rate of production minus rate of metabolism) of the 17-hydroxycorticosteroid pool per unit of time (254). As to the second technique, if the specific activity of one of the urinary metabolites (chromatographically separated) is determined, the rate of turnover per urine collection period can be calculated (255). Together with the determination of these specific activities, the con-

centration of the plasma or urinary fractions must be determined chemically or by the use of the double-isotope dilution derivative assay (254). The validity of such studies is based on several assumptions as enumerated by Peterson (254). These are: "(a) The injected steroid is pure. (b) The injected steroid does not alter appreciably the pool size and does not alter the metabolism of the endogenous steroid. (c) The pool remains constant throughout the period of study. (d) The rate of synthesis of the steroid equals the rate of transformation of the steroid. (e) The injected steroid is handled like the endogenous transformation of the steroid, and there is random disappearance of both the endogenous and the labelled steroid. (f) Mixing of the injected steroid within the pool is homogeneous and is rapid compared to its metabolism. (g) The rate of metabolism of the steroid is proportional to its concentration. (h) The isotopic steroid does not re-enter the pool. (i) The isolated steroid is pure."

By determining the specific activity of a urinary metabolite, Cope and Black have estimated that the 17-hydroxycorticosteroid production of the adrenal

glands is 4.9 to 27.9 mg. for 24 hours (255). With the plasma method, Peterson has been able to show that during salicylate therapy, the adrenocortical secretion rate remains normal; this had previously been uncertain (254). He has further shown that the adrenocortical secretion rate is reduced and that the plasma disappearance half time of the 17-hydroxycorticosteroids is prolonged with estrogen therapy; the converse was found in hyperthyroidism and during stress caused by the administration of bacterial pyrogens (254).

As to the advances made in the study of protein binding of the corticosteroids, Daughaday, employing reverse dialysis with 4-C^{14} labelled steroids, found 99% of cortisol is protein bound at the usual concentrations (256). The degree of binding among the various steroids studied was proportional to the number of hydroxyl groups in the molecule. Using electrophoresis and radioautography he found cortisol and corticosterone activity peaked at the region of the alpha globulins (257). Slaunwhite and Sandberg have confirmed this and have called the specific alpha globulin transcortin (258). More recently, Sandberg has demonstrated that transcortin binds cortisol 6,000

times as strongly as does albumin and that the concentration of transcortin increases progressively throughout pregnancy (259). Sandberg and Slaunwhite have more recently shown transcortin levels to be lower than normal in the newborn, in patients with the nephrotic syndrome and in some patients with multiple myloma; they are possibly elevated in the plasma of children and patients with rheumatoid arthritis (260). The plasma levels in cirrhotic patients were reported to be within normal limits. Furthermore, these authors felt that cortisol, which is bound to plasma proteins, is biologically inactive. They also observed that estrogen administration is followed by an elevation of the plasma transcortin levels. This latter observation has been confirmed by Wallace and Carter (261). This effect of estrogens possibly represents the mechanism whereby the 17-hydroxycorticosteroids of the plasma are elevated during the second half of pregnancy.

Mills, turning to another technique, one developed by Toribara in which ultra-filtrates are separated by centrifugal force, has shown that temperature affects the degree of binding (262). At 4°C. (the tempera-

ture at which Sandberg and Daughaday performed their dialyses) he found that 98% of the cortisol is protein bound whereas at body temperature, 37°C., only 94% is protein bound. Also, in vivo, he has shown that estrogens produce an increase in the degree of binding of cortisol.

Advances in the techniques of paper chromatography have been accompanied by increased knowledge as to the activity of these hormones. At the Laurentian Hormone Conference in 1954, Romanoff reported having isolated from human urine 32 different compounds which were either corticosteroids or their metabolic derivatives (263). In their chromatographic studies, Richardson, Touchstone and Dohan showed compounds E and F are freely interconvertible in Addisonian patients who have presumably little or no functioning adrenal tissue (264). The same convertibility was noted for compound A (dehydrocorticosterone) and compound B (corticosterone). Gold noticed that percentagewise the urinary THF increase in response to ACTH stimulation was greater than that of either compound F or THE (265). Dyrenfurth and others, using a Zaffaroni system for urinary extracts, compared the density of the various spots and found cortisone was normally

present in amounts which were about half those of cortisol and the THE and THF were, respectively, present in amounts 3 and 4 times that of cortisol (266). They found small amounts of 5 other uncharacterized steroids. In one of their normal subjects, they found compound B and in two they isolated the reduced metabolite of compound B.

A noteworthy application of the chromatography of extracts of urine and the incubates of adrenal tissue homogenates was undertaken by Bongiovanni and Eberlein (267, 268). They observed in cases of the adrenogenital syndrome, which had previously been described as showing high urinary 17-ketosteroids and low urinary 17-hydroxycorticosteroids, that, if they were normotensive, larger than normal quantities of 3, 17, 20-pregnantriol ("pregnantriol") and reduced amounts of steroids with an oxygen function at C-21 were extractable. Those cases which were also hypertensive lacked adrenocortical steroids with an oxygen function at C-11 so that they were found to have the reduced metabolites of 11-desoxycorticosterone and compound S (17-hydroxy-11-desoxycorticosterone). Normally, these steroids are not present in significant quantities. By applying these observations to Hechter's

scheme for bio-synthesis of the corticosteroids from progesterone they concluded that, in those cases with abnormal amounts of pregnantriol alone, there was impairment of hydroxylation at C-21 and, in the cases with hypertension, there was impairment of hydroxylation at C-11 as well as at C-21.

Many problems still remain. In the future we should expect to see the development of methods by which the tissue distribution of the corticosteroids can be accurately studied. The anatomic and biochemical sites of action of these hormones have yet to be defined. There are the problems of hormone interaction and hormone-enzyme interaction. The direct and secondary effects on protein, carbohydrate and fat metabolism will have to be more clearly understood. When such actions have been defined, we should be able to apply the knowledge to the control and correction of extra-adrenocortical abnormalities, deficiencies and blocks.

SECTION II

INVESTIGATION

A. ORGANIZATION

The protocol for the ACTH adrenocortical stimulation test, which is currently employed at the Royal Victoria Hospital, was established in 1955. It involves a measurement of the adrenocortical responsiveness to highly purified ACTH, 8 injections of 20 units, each administered intramuscularly over two days, as determined by the change in the urinary 17-hydroxycorticosteroids and 17-ketosteroids as well as the plasma 17-hydroxycorticosteroid level. The details as to methods and protocol will be found below in this section under, respectively, Chemical Methods and Experiments and Results. By 1956, close to 100 tests had been performed. About one fifth showed unexplained discrepancies which were beyond the limits of error for the methods. A lack of correlation between the levels of the plasma 17-hydroxycorticosteroids as well as those of the urine was obvious. Furthermore, some patients with definite adrenocortical hyperfunction were certainly not hyperresponsive by these parameters. The normal controls were few but the so-called normal

responses of patients indicated wide variability. Attempts to uncover some of the reasons behind these vagaries were undertaken in 1956.

A need for a clearer definition of normal levels and responsiveness was evident. With the cooperation of medical students, house officers and members of the nursing service, we were able to perform ACTH tests on 16 normal males and 6 normal females, all in the third and fourth decade of life. The means and standard deviations for the males and females were calculated. Similar calculations were done on 4 males and 14 females with adrenocortical hyperfunction secondary to proven or probable adrenocortical hypertrophy and the values were statistically compared to those of the normal controls.

An indirect assessment of the influence of renal function upon the plasma levels of the total free and the total glucuronide fractions was made. This involved the determination of these fractions in normal patients and subjects as well as in patients with chronic renal disease and acute renal failure. The means and standard deviations of the various groups were calculated and compared. These plasma fractions were also followed

in a few cases during their recovery from acute renal failure.

The in vivo use of radioisotope labelled hormones was not available to our laboratory in 1956, so that the study of the hormone pool size, disappearance rates and turnover rates was not feasible. Daughaday, however, had recently reported his methods for the determination of the dialysable and nondialysable fractions of the free and glucuronide 17-hydroxycorticosteroids together with their renal clearances (228). It was decided to adapt his methods to the existing laboratory facilities and to study these plasma fractions and their clearances under various doses of hydrocortisone and ACTH. The methods were set up and an evaluation of their precision and accuracy appears under Experiments and Results. Studies were performed on 6 normal subjects during ACTH tests, a cortisol disappearance study, cortisol infusion at a constant rate, and an ACTH infusion at a constant rate. Similarly, one patient with adrenocortical hyperfunction was studied during an ACTH test and again during a cortisol disappearance test. Also these techniques were employed on 3 totally adrenalectomized patients during constant infusions of cortisol and cortisol

with ACTH. All hormone clearances were compared to either an endogenous creatinine clearance or an insulin clearance. In certain cases paraaminohippuric acid and uric acid clearances were also performed for reference.

Two preliminary studies are also reported here. One was the measurement of the effect of a large dose of ACTH upon the rate of cortisol reduction and conjugation following cortisol loading, as determined by changes in the free and glucuronide fractions of the 17-hydroxy-corticosteroids. The other is a study in the feasibility of measuring the free-17-hydroxycorticosteroid content of the protein fractions of a 10 ml. serum specimen following exhaustive dialyses and starch gel electrophoresis. Large sample starch gel electrophoresis of serum is still under development.

B. CHEMICAL METHODS

1) The Routine Determination of the Urinary 17-Hydroxycorticosteroids.

The routine urinary 17-hydroxycorticosteroids for the ACTH stimulation tests were determined by a modification of the method originally described by Porter and Silber (133). Enzyme hydrolysis was performed prior to extraction and part of the extraction was performed at

pH 1. A 25 ml. aliquot of urine was adjusted to pH 5 with glacial acetic acid. Thereafter, 15 mls. of pH 5 buffer solution, prepared from Colman acetate buffer tablets, was added. Following the addition of 7,500 units of β-glucuronidase, derived from beef liver, the mixture was incubated at 37°C. for 24 hours.

For extraction, the mixture was transferred to a separatory funnel with the aid of 60 mls. of distilled water. It was extracted twice with 50 mls. of redistilled chloroform. The aqueous phase was then acidified to pH 1 and extracted once again with 50 mls. of chloroform. The chloroform extracts were combined. From here on the method of Porter and Silber was followed. This included washing with 0.1 N. sodium hydroxide and distilled water and the determination of the phenylhydrazine chromagens by the blank correction technique. With these modifications, one is able to determine the total (free plus conjugated fractions) 17-hydroxycorticosteroids. Hydrocortisone was used as the standard. Recovery studies on free hydrocortisone and cortisone have been of the order of 95%. The index of precision for the total 17-hydroxycorticosteroid content, however, is less accurate as evidenced

by the fact that duplicate determinations have shown an agreement error of the order of 20%. Recoveries on the reduced conjugates of the 17-hydroxycorticosteroids have not been carried out because of their lack of availability. These determinations were done with the technical assistance of Mr. Vladamir Kazmin and Mrs. Eleanor Wallace and their co-workers.

2) The Routine Determination of the Plasma 17-Hydroxycorticosteroids.

The free-17-hydroxycorticosteroids of the plasma were determined according to the method of Busch and Silber (153). When it was desirable to determine the glucuronide fraction of the plasma the following modification was employed subsequent to the extraction of the free fraction: The aqueous phase was adjusted to pH 4.5 with 5% sulfuric acid. An equal volume of pH 5 acetate buffer was added. β-glucuronidase was added, 100 units every 2 hours for 3 times, and the incubation was performed at 37°C. for 24 hours. Following this the aqueous phase was extracted with methylene dichloride as per the Busch-Silber method. The entire hydrolysis was repeated for another 24 hours and this was followed by extraction. The methylene dichloride extracts were combined. Recoveries

of hydrocortisone added to plasma have been of the order of 90 to 108%. Duplicate determinations of the glucuronides have agreed within 10 to 20%. These determinations were performed with the technical assistance of Mrs. Eva Schoning and Miss Judith Pasztor.

3) The Determination of the Free and Glucuronide Fractions of the 17-Hydroxycorticosteroids in Plasma, Dialysate and Urine for Renal Clearance Studies as modified from the method of Daughaday (228) (See flow diagram, Figures I-a and I-b)

The dialysis of heparinized plasma was started within an hour of the drawing of the blood specimen. A 20 ml. aliquot of plasma was placed within a cellophane casing (Nojax 18/32 casing) which had been cleaned by boiling and rinsing in distilled water. The casing was tied to the hub of a mixing motor (60 RPM) and suspended into a cylinder containing 80 mls. of Krebs phosphate buffer, pH 7.4. Dialysis was performed at 4°C. for 48 hours. At the end of the period the volumes within and outside of the cellophane were carefully determined. Then they were transferred to test tubes, with the aid of 5 to 10 mls. of distilled water, for freezing and storage. Urine was also temporarily kept at 4°C. for 48 hours prior to freezing.

FIGURE I a

Flow Diagram of Steps in the Determination of the Free-17-Hydroxycorticosteroids of the Urine, Dialysate and Dialyzed Plasma

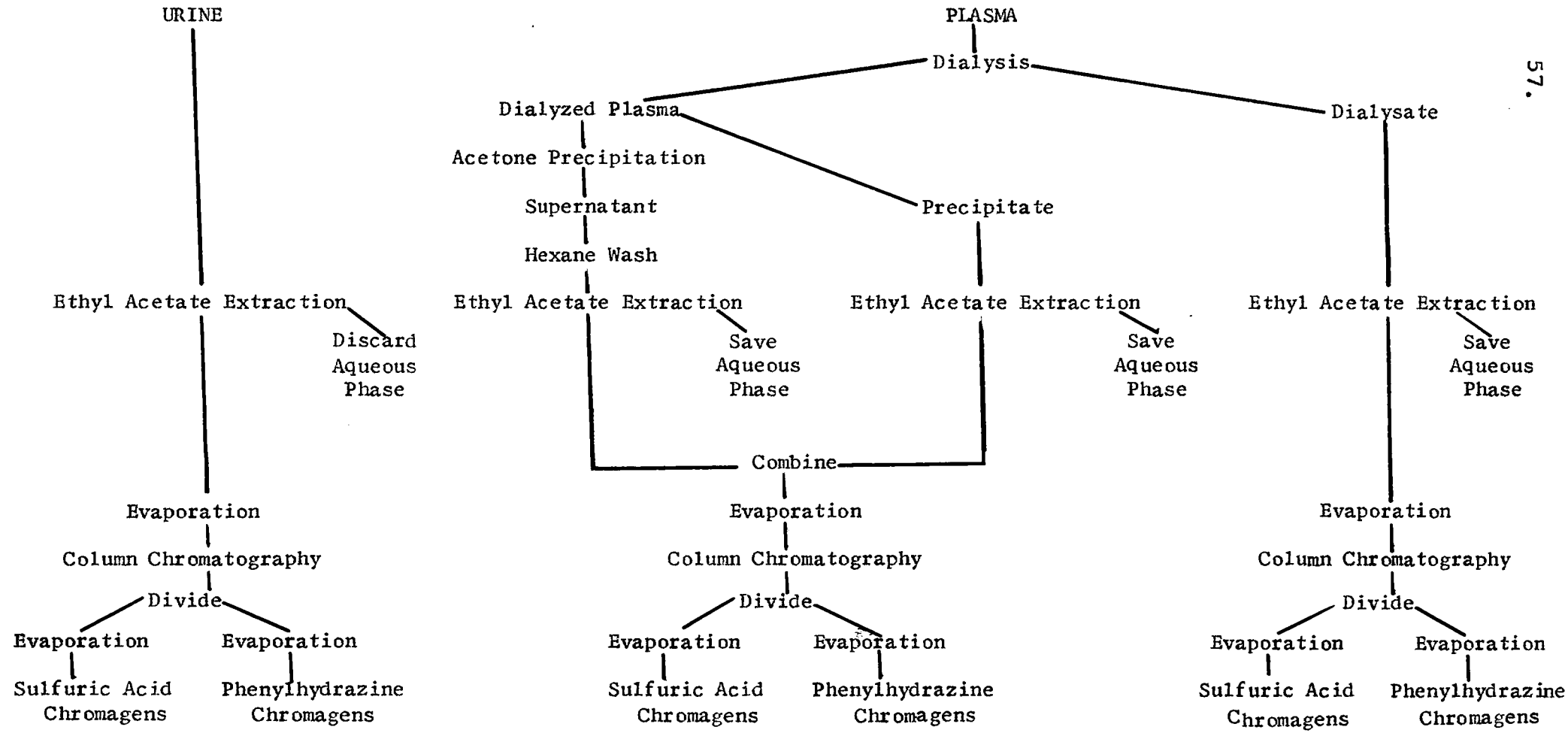
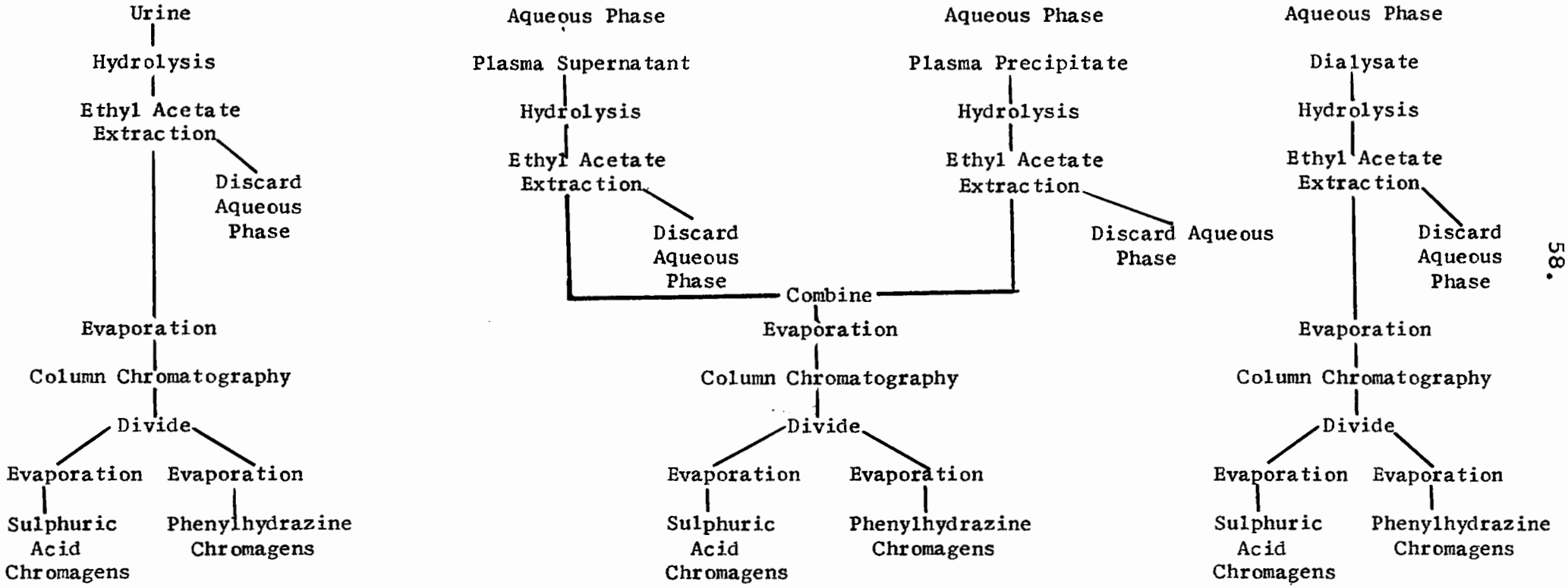


FIGURE I b

Flow Diagram of Steps in the Determination of the Total Urinary 17-Hydroxycorticosteroids and the 17-Hydroxycorticosteroid- Glucuronides of the Dialysate and Dialyzed Plasma



Prior to extraction, only the plasma was prepared in the following way: The proteins were precipitated with 4 volumes of cold freshly distilled acetone and this preparation was kept at 4°C. for 2 hours. The precipitate was separated by passage through filter paper then dissolved in 40 mls. of distilled water and saved. The acetone was evaporated from the supernatant under air pressure in a water bath at 45°C. The lipids were then removed from the supernatant by a hexane partition, 2 and 1/2 volumes of hexane were employed twice. The hexane was backwashed with 1/20th the volume of distilled water which was recombined with the aqueous phase of the supernatant.

The free-17-hydroxycorticosteroids were extracted from the plasma supernatant, plasma proteins, dialysate and urine with 1 and 1/2 volumes of ethyl acetate, three times. The minimum volume for the aqueous phase was 20 mls. The extracts of the plasma supernatant, plasma proteins and dialysate were backwashed with 1/10th the volume of distilled water and combined with the respective aqueous phase. The aqueous phase of urine was discarded following extraction. The extracts of the plasma super-

natant and plasma proteins were comtined. All extracts were evaporated to dryness under air pressure in a water bath at 45°C.

Enzyme hydrolysis was carried out on the aqueous phase of the plasma supernatant, plasma proteins and dialysate, which were saved following the above extraction, in preparation for the determination of the glucuronide fraction. A new aliquot of urine of a volume ranging from 0.5 mls. to 20 mls. was similarly hydrolyzed in preparation for the determination of the total 17-hydroxy-corticosteroids. Details of the hydrolysis are as follows: The pH was adjusted to 4.5 with 5% sulfuric acid and buffered with 1/4th the volume of quadruple strength acetate buffer, pH 5. β-glucuronidase was added, 50 units per each 10 mls. of aliquot or fraction thereof, every 2 hours for three times. Following incubation at 37°C. for 24 hours, extraction was performed as above except that backwashing was eliminated. This entire incubation and extraction procedure was repeated for another 24-hour period. All ethyl acetate extracts of the incubates were combined. Extracts of plasma supernatant and plasma proteins were combined.

Florisil column chromatographic partition was performed. It was necessary to prepare the Florisil most carefully. It was washed with distilled water three times and dried. Following this, it was twice washed in absolute alcohol and dried. Finally, it was washed in chloroform, which was freshly redistilled over calcium carbonate, and dried immediately. Before storage in brown glass bottles it was heated to 120°C. for 3 days. The day prior to its use, it was heated to 600°C. ($\pm$25 degrees) for 3 and 3/4 to 4 hours and thereafter kept in a desiccator until it was used. Columns with an internal diameter of 11 mm. were employed. Stoppers of glass wool, which had been twice washed in chloroform, were placed at the bottom and top of the column respectively at the beginning and end of packing. The Florisil was tightly packed in chloroform in the columns for a distance of 7 cm. with the aid of a footed glass rod. This required about 5 grams of Florisil. It was kept under chloroform for the remainder of its use. The columns were run at a rate of 2 mls. per minute. The samples were transferred to the columns in 25 mls. of chloroform (freshly redistilled over calcium carbonate)

in 5 fractions of 5 mls. each. The second eluate of 25 mls. of 2% absolute methyl alcohol in freshly redistilled chloroform was discarded. The third eluate of 25% absolute methyl alcohol in freshly redistilled chloroform contained the corticosteroids. This was saved and divided equally. Thereafter, it was evaporated to dryness under air pressure in a water bath at 45°C. in test tubes with a conical shaped bottom. The steroids were concentrated at the bottom of the tube by twice washing the sides with 0.5-1.0 mls. of chloroform and following this with re-evaporation.

The phenylhydrazine reagent was prepared freshly each time as follows: 0.0108 grams of crystalline phenylhydrazine, 8.33 mls. of ketone free ethyl alcohol and 16.66 mls. of 62% sulfuric acid were thoroughly mixed together in a 25 ml. volumetric flask. The phenylhydrazine was omitted from the reagent which was used for the blanks. To one series of tubes 0.5 mls. of phenylhydrazine reagent was added and to the other series of tubes (the blanks) 0.5 mls. of the ethyl alcohol-sulfuric acid solution was added. All tubes were heated in a water bath at 60°C. for 30 minutes, cooled and their optical density measured

quickly. The optical density was determined at 410 mμ. in a Beckman DU spectrophotometer using ultraviolet light. All samples and standards were read against the ethyl alcohol-sulfuric acid reagent blank. The "true optical density" of the phenylhydrazine chromagens was calculated by subtracting that of the sulfuric acid chromagens of the respective sample blanks and the phenylhydrazine chromagens of the reagents from the uncorrected optical density of the phenylhydrazine chromagens of the samples.

Two sets of both reagent blanks and standards (5 and 10 μg. of hydrocortisone) were carried throughout each assay; one set was used in the determination of the free fraction, the other for that of the glucuronides.

The concentration of the nonprotein-bound hormone moiety was calculated as per the following formula:

$$P_{S-D} = \frac{D_S \times (V_C + V_D)}{V_P}$$

Where:

P_{S-D} = The concentration of the dialysable hormone moiety (nonprotein bound) in the original plasma sample.

D_S = The concentration of the hormone in the dialysate.

V_C = The final volume of the contents of the cellophane casing.

V_D = The final volume of the dialysate.

V_P = The original volume of the plasma sample.

Similarly, the concentration of the protein-bound moiety was determined by employing the following formula:

$$P_{S-ND} = \frac{R_{S-T} - (D_S \times V_C)}{V_P}$$

Where:

P_{S-ND} = The concentration of the nondialysable hormone moiety (protein bound) in the original plasma sample.

R_{S-T} = The total amount of the hormone within the casing.

D_S = As shown above.

V_C = As shown above.

V_P = As shown above.

The urinary glucuronide fraction was determined by subtracting the urinary free fraction from the total

urinary 17-hydroxycorticosteroids. The modifications from the method originally described by Daughaday are as follows: The dialysis was performed in a cylinder with the cellophane casing tied to a motor instead of in tubes rotating on a disc. Hexane was used in place of the Skelly Solve B and before the acetone precipitation instead of afterwards. The precipitated plasma proteins as well as the plasma supernatant were extracted. The binding affinity of the protein content of the plasma samples was not determined. These determinations were done by the author with the technical assistance of Mr. George Ajemian.

4) The Determinations of the Creatinine Content of the Plasma and the Urine.

The method of Owen, Iggo, Scandrett, and Stewart was employed (269). Recoveries were of the order of 76 to 114%. These determinations were done by Miss Geraldine Sullivan.

5) The Determination of the Inulin Content of the Plasma and the Urine.

Schreiner's modification of the method originally described by Roe, Epstein and Goldstein was used for the determination of the inulin levels of the plasma

and urine (270). Recoveries ranged from 98 to 102%. These determinations were done by Miss Audrey Jardine.

6) The Determination of the Para-aminohippuric Acid Content of the Plasma and the Urine.

The modification developed by Smith from that originally described by Braton and Marshal was employed to determine the concentrations of PAH in the plasma and the urine (271). Recoveries were in the neighborhood of 98 to 103%. These determinations were done by Miss Audrey Jardine.

7) The Determination of the Uric Acid Content of Plasma and Urine.

The modification of Folin's original method as set forth by Norwood was used for the determination of the uric acid content of the plasma and the urine (272). Recoveries on urine samples ranged from 99.8% to 101%. On serum samples recoveries ranged between 100% and 102%. These determinations were carried out by Miss Joan Ohashi.

8) The Method of Starch Gel Zone Electrophoresis of Dialyzed Serum.

This was performed by a method of "large sample" starch gel electrophoresis which is currently under development by Dr. James H. Pert of the American Red Cross. As yet, it is not quantitatively accurate. An

aliquot of 10 to 15 mls. of serum can be used. The original description of starch gel electrophoresis, which was described by Smithies, employed as little as 0.02 mls. of serum (273). This determination was performed by Dr. James Pert in the laboratory of Dr. Oliver Smithies, Connaught Medical Research Laboratories, Toronto.

C. EXPERIMENTS AND RESULTS

1) ACTH Tests.

a) The routine protocol of the Endocrine Service of the Royal Victoria Hospital for ACTH tests was employed. Urine was collected in 24-hour specimens, 8 a.m. to 8 a.m. on days 1 through 6. ACTH (Acton X, Nordic Biochemicals Ltd., Montreal), 20 units, was administered intramuscularly as follows: at 8 a.m., 12 p.m. and 6 p.m. on day 3; 12 a.m., 6 a.m., 12 p.m. and 6 p.m. on day 4 and 12 a.m. on day 5. Heparinized blood was drawn on day 3 at 8 a.m. and 12 p.m., just prior to the ACTH injections, and on day 5 at 8 a.m. The total urinary 17-hydroxycorticosteroids was determined by the beforementioned modification of the Porter-Silber method. The average level for days 1 and 2 served as a control. The levels on days 3 through 6 were a measure of the

adrenocortical responsiveness of the subject. The free-17-hydroxycorticosteroids of the plasma were determined by the Busch-Silber method previously referred to. The value for 8 a.m. on day 3 served as a control and those drawn at 12 p.m. on day 3 and 8 a.m. on day 5 similarly reflected the adrenocortical responsiveness.

b) The Experimental Subjects: Six females, graduate nurses and house officers between the ages of 22 and 35 years, served as female controls. The 16 male controls were medical students and house officers ranging in age from 22 to 34 years. All control subjects performed their routine hospital duties during the 6 days; they were, however, requested not to engage in strenuous physical activity during this period. The 14 female cases of adrenocortical hyperfunction were patients followed on the Endocrine Service of the Royal Victoria Hospital. Their clinical findings varied from those of obesity, hirsutism and amenorrhea to frank Cushing's syndrome with striae, hypertension, osteoporosis and mild diabetes mellitus. Cases numbered 1, 4, 6, 7, 8, 9 and 14 were shown following bilateral adrenalectomy, to have adrenocortical hyperplasia. Case 10 appeared to have unilateral

adrenocortical hypertrophy on the left and Cases 2, 3, 5, 13, 11 and 12 were diagnosed on clinical findings; retroperitoneal carbon dioxide insufflation studies and nephrotomography were employed in the evaluation of most cases. The 4 men with adrenocortical hyperfunction were from the same medical service and were all fairly typical examples of Cushing's syndrome. Cases numbered 1, 2 and 3 were shown, following bilateral adrenalectomy, to have bilateral adrenocortical hyperplasia. Case 3 was later shown at craniotomy to have an adenocarcinoma of the anterior lobe of the pituitary. Case 4 has been diagnosed on clinical grounds. All patients with adrenocortical hyperfunction were hospital in-patients during their ACTH tests and most were ambulatory at the time.

c) Results of the Chemical Methods: The results of the ACTH tests are listed in Tables I through VIII. Table I contains the urinary findings in the 6 normal females. Part "a" presents the results as mg./24 hours. Part "b" shows the change from the control level in mg./24 hours. Part "c" lists the change as percentage deviation from the control level per 24-hour specimen. Table V lists the free-17-hydroxycorticosteroids of the

plasma at 0, 4 and 48 hours of ACTH administration in µg./100 mls. Part "b" gives the change from the control level at 0 hours which was found at 4 and 48 hours and expressed as µg. change/100 mls. Part "c" expresses these changes as percentage deviation from the control level. Tables II and VI similarly give the results for females with adrenocortical hyperfunction. Tables III and VII give the results for the normal males and Tables IV and VIII those of the males with adrenocortical hyperfunction.

d) Statistical Methods: The means of the various specimens in each group of subjects have been calculated and the standard deviation for each mean is listed directly below it. The standard deviation was calculated according to the following formula as mentioned by Mainland (274).

$$S.D. = \sqrt{\frac{S(X-\bar{X})^2}{N-1}}$$

Where:

S.D. = Standard Deviation
S = Sum
$\underline{X}$ = Any one observation
$\bar{X}$ = Mean of all observations
N = Number of observations

The statistical comparison of the results of the adrenocortical hyperfunction cases with those of the normal controls was accomplished by the use of the "T-Test" (274). The T value was calculated from the following formula:

$$T = \frac{\text{Difference between means}}{\text{Standard Deviation}} \sqrt{\frac{N_1 N_2}{N_1 + N_2}}$$

Where:

N_1 = Number of observations in one series

N_2 = Number of observations in the other series

Following the determination of the T value, the P value (probability value) was determined by using the Student's "T" Distribution Table. Any P value of .05 or less was considered significant.

e) Results of Statistical Analysis: The urine values for the 6 normal females show a mean control level of 3.9 $\pm$ 0.8 mg./24 hours. The mean response is greatest on day 4 with a mean increase of 20.7 $\pm$ 8.2 mg./24 hours over and above the control level. The subject J.B. had her greatest response on day 3. The mean increase on day 4 percentage-wise was 549% $\pm$ 228% of the control value. In comparison the mean control level for the females with adrenocortical hyperfunction was 8.9 $\pm$ 2.2 mg./24 hours which was significantly elevated. The

mean increase was again greatest on day 4 at 22.3 ± 14.7 mg./24 hours which was within normal limits. The mean percentage increase was 186%, 281% and 25% on days 3, 4 and 5; these values are significantly reduced from normal. The mean values for the plasma 17-hydroxycorticosteroids in the 6 female controls at 0, 4 and 48 hours were 13.7 ± 6.4 µg./100 mls., 31.2 ± 12.8 µg./100 mls. and 37.9 ± 30.1 µg./100 mls. respectively. J.B.'s and H.B.'s plasma values at 48 hours were lower than at 4 hours. The corresponding values as well as both the µg. increase/100 mls. and the percentage increase of the plasma values in the females with adrenocortical hyperfunction were statistically normal.

The urine values in the normal males revealed a control level of 6.1 ± 2.1 mg./24 hours. Again the greatest mean response was observed on day 4 at an increase of 30.4 ± 16.5 mg./24 hours over the baseline. The corresponding mean percentage increase was 519% ± 283%. The mean control value for the 4 males with adrenocortical hyperfunction was significantly elevated at 15.8 ± 7.3 mg./24 hours as were the mean values for days 5 and 6 at, respectively, 40.2 ± 12.9 mg./24 hours and 23.2 ± 12.0

TABLE Ia

ACTH Tests, Normal Females

Urinary 17-Hydroxycorticosteroid Content, mg./24 Hours.

Subject	Age	Day 1 & 2	Day 3	Day 4	Day 5	Day 6
C.C.	24	4.2	18.6	24.2	11.8	8.0
J.Y.	22	4.5	12.3	21.3	21.3	3.4
J.B.	22	3.6	15.9	12.4	2.8	1.7
J.O.	22	4.9	25.8	37.3	9.5	4.2
J.M.	35	3.1	20.1	30.2	8.1	4.5
H.D.	29	2.9	18.7	22.1	3.7	2.0
Mean		3.9	18.6	24.6	9.5	4.0
$\pm$ 1 S.D.		$\pm$.8	$\pm$4.5	$\pm$8.5	$\pm$6.7	$\pm$2.3

TABLE Ib

ACTH Tests, Normal Females

Urinary 17-Hydroxycorticosteroid Content, mg. Change/24 Hours.

Subject	Day 3	Day 4	Day 5	Day 6
C.C.	14.4	20.0	7.6	3.8
J.Y.	7.8	16.8	16.8	-1.1
J.B.	12.3	8.8	-.8	-1.9
J.O.	20.9	32.4	4.6	- .7
J.M.	17.0	27.1	5.0	1.4
H.D.	15.6	19.2	.8	- .9
Mean	14.7	20.7	5.7	.1
± 1 S.D.	±4.4	±8.2	±6.2	±2.0

TABLE Ic

ACTH Tests, Normal Females

Urinary 17-Hydroxycorticosteroid Content, o/o Change/24 Hours.

Subject	Day 3	Day 4	Day 5	Day 6
C.C.	343	476	181	91
J.Y.	173	373	373	-24
J.B.	342	244	-22	-53
J.O.	427	661	94	-15
J.M.	548	874	161	45
H.D.	538	662	28	-31
Mean	395	549	136	2
± 1 S.D.	±141	±228	±201	±55

TABLE II a

ACTH Tests, Females with Adrenocortical Hyperfunction

Urinary 17-Hydroxycorticosteroid Content, mg./24 Hours.

Case	Age	Day 1 & 2	Day 3	Day 4	Day 5	Day 6
1	60	9.6	18.7	16.9	16.2	3.1
2	24	6.7	26.2	28.0	5.8	7.9
3	23	6.6	28.6	41.4	11.4	6.2
4	19	11.5	17.3	39.6	12.8	10.6
5	23	8.3	26.1	24.1	8.1	4.7
6	26	6.8	9.6	7.9	2.5	5.1
7	20	9.6	21.5	30.1	20.1	4.8
8	25	5.9	17.3	25.4	1.5	4.1
9	33	8.6	42.9	56.8	17.1	2.9
10	19	10.4	34.3	31.5	13.0	11.9
11	40	10.0	8.7	25.1	10.1	10.9
12	16	10.5	49.4	31.3	11.9	14.4
13	19	7.0	16.6	38.8	13.5	8.0
14	29	13.6	26.5	31.2	15.9	9.3
Mean		8.9	24.6	30.6	11.4	7.4
± 1 S.D.		±2.2	±11.6	±11.3	± 5.4	± 3.6
			.30>	.25>	.50>	.10>
P value		P < .001	P > .20	P > .20	P > .40	P > .05

TABLE II b

ACTH Tests, Females with Adrenocortical Hyperfunction

Urinary 17-Hydroxycorticosteroid Content, mg. Change/24 Hrs.

Case	Day 3	Day 4	Day 5	Day 6
1	9.1	7.3	6.6	-6.5
2	19.5	21.3	-.9	1.2
3	22.0	34.8	4.8	- .4
4	5.8	28.1	1.3	- .9
5	17.8	15.8	- .2	-3.6
6	2.8	1.1	-4.3	-1.7
7	11.9	32.4	10.5	-4.8
8	11.4	30.9	-4.4	-1.8
9	34.3	48.2	8.5	-5.7
10	23.9	21.1	2.6	1.5
11	-1.3	15.1	.1	.9
12	39.4	20.8	1.4	3.9
13	9.6	31.8	6.5	1.0
14	12.9	17.6	2.3	-4.3
Mean	15.7	23.3	2.5	-1.5
$\pm$ 1 S.D.	$\pm$11.5	$\pm$14.7	$\pm$4.5	$\pm$3.1
P value	.10>P>.05	.80>P>.70	.20>P>.10	.20>P>.10

TABLE II c

ACTH Tests, Females with Adrenocortical Hyperfunction

Urinary 17-Hydroxycorticosteroid Content, o/o Change/24 Hrs.

Case	Day 3	Day 4	Day 5	Day 6
1	95	76	69	-68
2	291	318	-13	18
3	334	528	72	- 6
4	50	244	11	- 8
5	225	191	- 2	-43
6	41	16	-63	-25
7	124	345	109	-50
8	193	524	-75	-31
9	399	561	99	-66
10	230	202	25	14
11	-13	151	1	9
12	380	198	13	37
13	137	454	93	14
14	95	130	17	-32
Mean	186	281	25	-17
± 1 S.D.	±131	±178	±65	±33
P value	.01>P>.001	.01>P>.001	.01>P>.001	.30>P>.20

TABLE III a

ACTH Tests, Normal Male

Urinary 17-Hydroxycorticosteroid Content, mg./24 Hours

Subject	Age	Day 1 & 2	Day 3	Day 4	Day 5	Day 6
D.G.	27	6.5	52.8	39.3	22.7	2.8
R.M.	31	6.4	29.0	34.5	22.1	4.4
P.M.	25	4.5	19.9	44.6	16.1	6.7
A.B.	27	3.1	22.7	21.5	4.4	4.2
I.H.	28	5.2	27.2	21.7	19.7	7.6
S.H.	28	5.3	7.8	24.6	2.1	6.2
T.H.	26	5.4	17.0	17.4	12.1	6.0
R.D.	28	5.1	16.0	23.7	9.6	4.7
C.V.	28	5.7	19.2	19.0	3.4	2.0
N.D.	26	4.3	23.2	24.8	11.1	3.2
D.C.	24	5.4	18.6	20.6	5.0	3.3
A.M.	29	6.7	30.3	30.4	13.4	4.4
A.M.	29	6.3	26.6	47.2	16.7	4.4
W.B.	22	12.1	24.3	50.0	20.8	7.2
W.M.	24	10.4	31.7	41.8	13.0	8.7
D.B.	24	6.5	26.9	75.5	45.1	7.3
J.B.	34	5.5	45.9	66.4	22.2	6.9
J.B.	34	5.6	57.0	54.0	34.0	7.2
Mean		6.1	27.6	36.5	16.3	5.4
$\pm$ 1 S.D.		$\pm$2.1	$\pm$12.8	$\pm$17.8	$\pm$8.9	$\pm$1.9

TABLE III b

ACTH Tests, Normal Males

Urinary 17-Hydroxycorticosteroid Content, mg. Change/24 Hours

Subject	Day 3	Day 4	Day 5	Day 6
D.G.	46.3	32.8	16.2	-3.7
R.M.	22.6	28.1	15.7	-2.0
P.M.	15.4	40.1	11.6	2.2
A.B.	19.6	18.1	1.3	1.1
I.H.	22.0	16.5	14.5	2.4
S.H.	2.5	19.3	-3.2	4.1
T.H.	11.6	12.0	6.7	.6
R.D.	10.9	18.6	4.5	- .4
C.V.	13.5	13.3	-2.3	-3.7
N.D.	18.9	20.5	6.8	-1.1
D.C.	13.2	15.2	- .4	-2.1
A.M.	23.6	23.7	6.7	-2.3
A.M.	20.3	40.9	10.4	-1.9
W.B.	12.2	37.9	8.7	-4.9
W.M.	21.3	31.4	2.6	-1.7
D.B.	20.4	69.0	38.6	.8
J.B.	40.4	60.9	16.7	1.4
J.B.	51.4	48.4	28.4	1.6
Mean	21.5	30.4	10.2	- .5
±1 S.D.	±12.6	±16.5	±10.8	±2.5

TABLE III c

ACTH Tests, Normal Males

Urinary 17-Hydroxycorticosteroid Content, o/o Change/24 Hours

Subject	Day 3	Day 4	Day 5	Day 6
D.G.	712	505	249	-57
R.M.	353	439	245	-31
P.M.	342	891	258	49
A.B.	632	594	41	35
I.H.	423	317	279	46
S.H.	47	364	-60	77
T.H.	215	222	124	11
R.D.	214	365	88	- 8
C.V.	237	233	-40	-65
N.D.	440	477	158	-26
D.C.	244	282	- 7	-39
A.M.	352	354	0	-34
A.M.	322	649	165	-30
W.B.	101	313	72	-40
W.M.	205	302	25	-16
D.B.	314	1062	593	12
J.B.	735	1107	304	25
J.B.	918	864	507	29
Mean	378	519	167	- 3
± 1 S.D.	±237	±283	±176	±39

TABLE IV a

ACTH Tests, Males with Adrenocortical Hyperfunction

Urinary 17-Hydroxycorticosteroid Content, mg./24 Hrs.

Case	Age	Day 1 & 2	Day 3	Day 4	Day 5	Day 6
1	39	7.3	27.1	47.9	56.3	29.3
2	57	18.8	30.0	40.4	44.7	34.8
3	24	24.2	25.9	40.4	29.7	21.7
4	30	13.0	62.7	87.1	29.9	7.1
Mean		15.8	36.4	54.0	40.2	23.2
± 1 S.D.		±7.3	±16.7	±22.6	±12.9	±12.0
		.05 >	.40 >	.20 >	.01 >	.01 >
P value		P	P	P	P	P
		> .02	> .30	> .10	> .001	> .001

TABLE IV b

ACTH Tests, Males with Adrenocortical Hyperfunction

Urinary 17-Hydroxycorticosteroid Content, mg. Change/24 Hrs.

Case	Day 3	Day 4	Day 5	Day 6
1	19.8	40.6	49.0	22.0
2	11.2	21.6	25.9	16.0
3	1.7	16.2	5.5	-2.5
4	49.7	74.1	16.9	-5.9
Mean	20.6	38.1	24.3	7.4
± 1 S.D.	±20.7	±26.2	±18.4	±13.7
P value	P > .90	P=.60	.05>P>.02	.10>P>.05

TABLE IV c

ACTH Tests, Males with Adrenocortical Hyperfunction

Urinary 17-Hydroxycorticosteroid Content, o/o Change/24 Hrs.

Case	Day 3	Day 4	Day 5	Day 6
1	272	556	672	301
2	60	115	138	85
3	7	67	23	-10
4	383	571	130	-45
Mean	180	327	241	83
± 1 S.D.	±177	±273	±292	±155
P value	.10>P>.05	.25>P>.20	.50>P>.40	.10>P>.05

TABLE V a

ACTH Tests, Normal Females

Plasma 17-Hydroxycorticosteroid Content µg./100 mls.

Subject	0 Hrs.	4 Hrs.	48 Hrs.
C.C.	7.5	20.0	31.0
J.Y.	22.0	32.0	12.5
J.B.	13.5	14.0	21.0
J.O.	8.0	34.0	42.0
J.M.	21.0	50.0	96.0
H.B.	10.0	37.0	25.0
Mean	13.7	31.2	37.9
± 1 S.D.	±6.4	±12.8	±30.1

TABLE V b

ACTH Tests, Normal Females

Plasma 17-Hydroxycorticosteroid Content, µg. Change/100 mls.

Subject	4 Hrs.	48 Hrs.
C.C.	12.5	23.5
J.Y.	10.0	-9.5
J.B.	0.5	7.5
J.O.	26.0	34.0
J.M.	29.0	75.0
H.D.	27.0	15.0
Mean	17.5	24.3
± 1 S.D.	±11.5	±28.9

TABLE V c

ACTH Tests, Normal Females

Plasma 17-Hydroxycorticosteroids, o/o Change

Subject	4 Hrs.	48 Hrs.
C.C.	167	313
J.Y.	46	-43
J.B.	4	56
J.O.	325	425
J.M.	131	357
H.D.	270	150
Mean	157	210
± 1 S.D.	±125	±185

TABLE VI a

ACTH Tests, Females with Adrenocortical Hyperfunction

Plasma 17-Hydroxycorticosteroid Content, µg./100 mls.

Case	0 Hrs.	4 Hrs.	48 Hrs.
2	17.0	12.0	14.0
3	19.5	21.5	57.5
4	19.0	35.0	23.0
5	21.0	37.0	70.0
6	8.5	33.0	20.0
7	10.0	35.0	55.0
8	6.0	24.0	30.0
9	23.0	33.0	44.0
10	19.0	27.5	32.0
11	20.0	24.0	23.0
12	6.9	25.9	16.5
13	19.0	39.5	53.0
14	17.0	34.0	68.0
Mean	15.8	29.3	38.9
± 1 S.D.	±5.8	±7.6	±19.3
P Value	.50>P>.40	.70>P>.60	P>.90

TABLE VI b

ACTH Tests, Females with Adrenocortical Hyperfunction

Plasma 17-Hydroxycorticosteroid Content, µg. Change/100 mls.

Case	4 Hrs.	48 Hrs.
2	-5.0	-3.0
3	2.0	38.0
4	54.0	4.0
5	16.0	49.0
6	24.5	11.5
7	25.0	45.0
8	18.0	24.0
9	10.0	21.0
10	8.5	13.0
11	4.0	3.0
12	19.0	9.6
13	20.5	34.0
14	17.0	51.0
Mean	16.4	23.1
± 1 S.D.	±14.5	±18.6
P Value	.90>P>.80	.90>P>.80

TABLE VI c

ACTH Tests, Females with Adrenocortical Hyperfunction

Plasma 17-Hydroxycorticosteroid Content, o/o Change

Case	4 Hrs.	48 Hrs.
2	-29	-18
3	10	195
4	584	21
5	76	233
6	289	135
7	250	450
8	300	400
9	44	91
10	44	68
11	20	15
12	275	494
13	108	179
14	100	300
Mean	159	197
$\pm$ 1 S.D.	$\pm$172	$\pm$170
P value	$P > .90$	$.90 > P > .80$

TABLE VII a

ACTH Tests, Normal Males

Plasma 17-Hydroxycorticosteroid Content, µg./100 mls.

Subject	0 Hrs.	4 Hrs.	48 Hrs.
D.G.	19.0	35.0	68.0
R.M.	13.0	41.0	57.0
P.M.	7.5	46.0	
A.B.	17.0	34.0	22.0
I.H.	27.5	39.0	16.0
S.H.	23.0	12.5	10.0
T.H.	15.0	35.6	16.0
R.D.	13.5	40.0	13.0
C.V.	15.0	28.0	
N.D.	10.0	42.0	50.0
D.G.	3.0	24.0	3.0
A.M.	28.0	37.0	45.0
A.M.		63.0	48.0
W.B.	7.5	45.0	20.5
W.M.	30.5	45.0	44.0
D.B.	8.5	14.0	56.0
J.B.	10.0	54.0	80.0
J.B.	13.0	44.5	57.0
Mean	15.4	37.8	37.8
± 1 S.D.	±7.9	±12.5	±23.4

TABLE VII b

ACTH Tests, Normal Males

Plasma 17-Hydroxycorticoid Content, µg. Change/100 mls.

Subject	4 Hrs.	48 Hrs.
D.G.	16.0	49.0
R.M.	28.0	44.0
P.M.	38.5	
A.B.	17.0	5.0
I.H.	11.5	-11.5
S.H.	-10.5	-13.0
T.H.	20.6	1.0
R.D.	26.5	- 0.5
C.V.	13.0	
N.D.	32.0	40.0
D.C.	21.0	0.0
A.M.	9.0	17.0
W.B.	37.5	13.0
W.M.	14.5	13.5
D.B.	19.0	47.5
J.B.	44.0	70.0
J.B.	31.5	44.0
Mean	20.2	21.3
± 1 S.D.	±13.3	±25.6

TABLE VII c

ACTH Tests, Normal Males

Plasma 17-Hydroxycorticoid Content, o/o Change

Subject	4 Hrs.	48 Hrs.
D.G.	84	260
R.M.	215	339
P.M.	513	
A.B.	100	29
I.H.	42	-42
S.H.	-46	-57
T.H.	137	7
R.D.	196	- 4
C.V.	87	
N.D.	320	400
D.C.	700	0
A.M.	32	61
W.B.	500	180
W.M.	62	156
D.B.	224	559
J.B.	444	700
J.B.	242	339
Mean	227	195
± 1 S.D.	±205	±232

TABLE VIII a

ACTH Tests, Males with Adrenocortical Hyperfunction

Plasma 17-Hydroxycorticosteroid Content, µg./100 mls.

Case	0 Hrs.	4 Hrs.	48 Hrs.
1	18.0	65.0	95.0
2	34.5	59.0	96.0
4	15.0	65.0	76.5
Mean	22.5	63.0	89.2
± 1 S.D.	±10.3	±3.5	±11.1
P value	.30>P>.20	P<.001	P<.001

TABLE VIII b

ACTH Tests, Males with Adrenocortical Hyperfunction

Plasma 17-Hydroxycorticoid Content, µg. Change/100 mls.

Case	4 Hrs.	48 Hrs.
1	47.0	77.0
2	24.5	61.5
4	50.0	61.5
Mean	40.5	66.7
± 1 S.D.	±13.9	±9.0
P value	.05>P>.02	P<.001

TABLE VIII c

ACTH Tests, Males with Adrenocortical Hyperfunction

Plasma 17-Hydroxycorticosteroid Content, o/o Change

Case	4 Hrs.	48 Hrs.
1	261	427
2	71	179
4	334	411
Mean	222	339
± 1 S.D.	±136	±139
P Value	P>.90	.20>P>.10

mg./24 hours. The milligram increases over the control level on days 5 and 6 were significantly elevated but percentagewise none of the increased urinary levels were significant. As for the mean plasma values of the male controls they were 15.4 ± 7.9 µg./100 mls., 37.8 ± 12.5 µg./100 mls. and 37.8 ± 23.4 µg./100 mls. at 0, 4 and 48 hours respectively. Out of 16 subjects, 7 showed a lower value at 48 hours than at 4 hours. As to the males with adrenocortical hyperfunction, their mean levels at 4 and 48 hours were significantly elevated; they were 63.0 ± 3.5 µg./100 mls. and 89.2 ± 11.1 µg./100 mls. At 0 hours, the mean level was normal at 22.5 ± 10.3 µg./100 mls. The mean microgram increase at these hours was also significantly elevated but percentagewise the increase was within normal limits. Dr. John Paul, Statistician for the Department of Genetics, McGill University, has concurred in the statistical methods and results.

2) Plasma 17-Hydroxycorticosteroid Levels in Renal Failure.

a) The Protocol: Heparinized blood was drawn at 8 a.m. on the normal controls and the patients with chronic renal disease. Specimens from the patients with acute renal failure were usually drawn in the morning;

some, however, were afternoon and evening specimens. Both the free-17-hydroxycorticosteroids and the 17-hydroxycorticosteroid-glucuronide levels were determined according to the Busch-Silber method and the noted modifications.

b) The Experimental Subjects: The normal controls were medical students, graduate nurses, house officers and unstressed patients with normal urinalyses and serum NPN content. The various types of chronic and acute renal impairment involved are listed in parts "b" and "c" of Table IX.

c) Results of the Chemical Methods: The results are listed in Table IX parts "a," "b" and "c."

d) Statistical Methods: The same statistical methods as those described in part 1 "d" of Experiments and Results were used.

e) Results of Statistical Analysis: A normal, control mean value of 16.1 $\pm$ 7.8 µg./100 mls. was found for the free-17-hydroxycorticosteroids while that for the 17-hydroxycorticosteroid-glucuronides was 19.9 $\pm$ 53.8 µg./100 mls. The means of the same fractions in the 7 patients with chronic renal disease are not significantly

different at the respective values of 14.8 $\pm$ 6.6 µg./100 mls. and 55.6 $\pm$ 54.0 µg./100 mls. In 11 cases of acute renal failure the means for both values were significantly elevated. The free-17-hydroxycorticosteroid level was 47.8 $\pm$ 27.0 µg./100 mls. and that of the 17-hydroxycorticosteroid-glucuronides was 380.1 $\pm$ 277.5 µg./100 mls.

3) Follow-up Studies on the Plasma 17-Hydroxycorticosteroid Levels in Selected Patients with Acute Renal Failure.

a) The Protocol: The same protocol as that used in Experiments and Results, part 2 "a," was employed.

b) Experimental Subjects: Certain of the cases listed in Table IX "c" were followed through recovery or treatment.

c) Results of the Chemical Methods: In Tables X, XI and XII the results of 3 patients followed into their recovery periods are listed. It is observed that as the NPN returned towards normal both the free-17-hydroxycorticosteroids and the 17-hydroxycorticosteroid-glucuronides did likewise.

Tables XIII, XIV and XV show the results obtained when 3 cases were studied both before and after dialyses on the artificial kidney. The free-17-hydroxycorticosteroids

TABLE IX a

Plasma 17-Hydroxycorticosteroid and 17-Hydroxycorticosteroid-Glucuronide Content in Subjects with Normal Renal Function

Subject	Sex	Age	Free-17-OHCS µg./100 mls.	17-OHCS-Glucuronide µg./100 mls.
1	M	72	8.0	46.5
2	M	74	10.0	15.0
3	M	27	19.5	9.0
4	M	24	7.5	25.0
5	M	55	20.0	*
6	M	65	7.0	*
7	M	24	30.5	9.0
7	M	24	17.5	3.0
7	M	24	21.5	10.0
7	M	24	21.0	*
7	M	24	19.0	9.0
8	M	55	16.6	24.1
9	M	35	10.5	4.7
10	M	51	21.7	3.7
11	M	34	13.0	10.0
11	M	34	12.5	6.0
12	F	30	19.5	104.0
13	F	25	20.9	*
14	F	28	10.4	*
Mean			16.1	19.9
± 1 S.D.			±7.8	±53.8

* High Background

TABLE IX b

Plasma 17-Hydroxycorticosteroid and 17-Hydroxycorticosteroid-Glucuronide Content in Cases with Chronic Renal Disease

Case	Diagnosis	Sex	Age	NPN mg./ 100 mls.	Free-17-OHCS µg./100 mls.	17-OHCS-Gluc. µg./100 mls.
1	Chronic Glomerulonephritis	M			11.6	31.8
2	Arteriolar Nephrosclerosis	M	70	100	18.1	109.8
2	Arteriolar Nephrosclerosis	M	70	100	16.2	174.0
3	Chronic Glomerulonephritis	M	25	100	3.6	29.8
3	Chronic Glomerulonephritis	M	25	144	8.4	22.9
4	Diabetic Glomerulosclerosis	F		133	14.6	69.7
5	Gout with Nephropathy	M		200	18.4	7.5
6	Probable Pyelonephritis	F	37	50	15.4	21.6
7	Probable Pyelonephritis	F	71		27.0	33.7
		Mean			14.8	55.6
		± 1 S.D.			±6.6	±54.0
		P Value			.70>P>.60	.20>P>.10

TABLE IX c

Plasma 17-Hydroxycorticosteroid and 17-Hydroxycorticosteroid-Glucuronide Content in Cases with Acute Renal Failure

Case	Diagnosis	Sex	Age	NPN mg./100 mls.	Free-17-OHCS µg./100 mls.	17-OHCS-Gluc. µg./100 mls.
1	Acute Renal Failure undetermined etiology	F	28	192	25.0	321.0
2	Acute Tubular Necrosis, circulatory collapse	M	61	180	33.0	208.0
3	Acute Tubular Necrosis, circulatory collapse	M	59	197	22.7	154.0
4	Acute Tubular Necrosis, circulatory collapse	M	50	102	77.5	111.5
5	Acute tubular Necrosis	M	46	292	59.0	604.0
6	Acute Renal Failure, undetermined etiology	M			93.7	766.0
7	Acute Exacerbation of Glomerulonephritis	F	42	240	16.6	107.1
8	Acute Exacerbation of Chronic Pyelonephritis	F	26	240	76.5	441.0
9	Renal Cortical Necrosis	M	46	265	24.4	209.0
10	Acute Tubular Necrosis, circulatory collapse	M	30	115	33.0	329.0
11	Acute Tubular Necrosis	F	49	195	64.5	930.0
Mean					47.8	380.1
± 1 S.D.					±27.0	±277.5
P Value					.01>P>.001	.01>P>.001

TABLE X

Case 1, Acute Renal Failure of Undetermined Etiology

General Supportive Care: Fluid Balance, Testosterone,

Neomycin Bladder Irrigations.

Date	NPN mg./100 mls.	Free 17-OHCS µg./100 mls. Plasma	17-OHCS-Gluc. µg./100 mls. Plasma
Mar. 24, 1958	192		
Mar. 28, 1958		25.0	321.0
Apr. 1, 1958	274		
Apr. 2, 1958		17.0	289.0
Apr. 5, 1958		32.5	280.0
Apr. 6, 1958	228		
Apr. 11, 1958	144		
Apr. 12, 1958		14.0	264.0
Apr. 17, 1958	75		
Apr. 18, 1958		4.0	104.0
Apr. 28, 1958	31		
May 13, 1958		4.5	20.0

TABLE XI

Follow-up

Case 7, Acute Exacerbation of Glomerulonephritis

General Supportive Care: Fluid Balance, Testosterone

Date	NPN mg./ 100 mls.	Free 17-OHCS µg./100 mls. Plasma	17-OHCS-Gluc. µg./100 mls. Plasma	Comment
Dec. 19, 1958	240			
Dec. 28, 1958	279.5			
Dec. 31, 1958	268	16.6	107.1	
Jan. 3, 1959	225	9.4	54.7	
Jan. 8, 1959	147			Start diuresis
Jan. 9, 1959	139	52.2	350.0	Cortisone 250 mg. q.d.
Jan. 13, 1959		40.8	335.0	Cortisone 250 mg. q.d.
Jan. 14, 1959	77			Cortisone 250 mg. q.d.
Jan. 16, 1959				Discontinue Cortisone
Jan. 17, 1959	67.6			
Jan. 20, 1959	50	6.4	10.3	
Feb. 2, 1959		6.4	*	
Feb. 9, 1959	102	7.5	*	
Feb. 12, 1959	67	8.5	*	

* High background, level less than 5 µg./100 mls.

TABLE XII

Follow-up

Case 10, Acute Tubular Necrosis with Circulatory Collapse

General Supportive Care: Fluid Balance, Tesosterone

Date	NPN mg./ 100 mls.	Free-17-OHCS µg./100 mls. Plasma	17-OHCS-Gluc. µg./100 mls. Plasma	Comment
Feb. 6, 1959	363			
Feb. 7, 1959	325	33.0	329.0	
Feb. 10, 1959	328	11.9	327.0	Start of diuresis
Feb. 12, 1959		24.8	126.0	
Feb. 14, 1959	115			
Feb. 15, 1959	75			
Feb. 16, 1959		17.3	14.3	End of diuresis

TABLE XIII

Follow-up

Case 9, Renal Cortical Necrosis

General Supportive Care: Fluid Balance, Tesosterone, Tetracycline

Date	NPN mg./ 100 mls.	Free-17-OHCS µg./100 mls. Plasma	17-OHCS-Gluc. µg./100 mls. Plasma	Comment
Jan. 6, 1959	225			
Jan. 9, 1959	265	24.4	209.0	Before dialysis
Jan. 9, 1959	193	37.1	168.0	During dialysis
Jan. 10, 1959	151.5	35.7	165.0	After dialysis
Jan. 10, 1959	121.5			Before dialysis
Jan. 10, 1959	106	29.1	139.0	After dialysis
Jan. 11, 1959	206.5	19.5	221.0	
Jan. 16, 1959	215	14.2	225.0	Before dialysis
Jan. 17, 1959	171	18.8	194.0	After dialysis
Jan. 19, 1959	171			
Jan. 22, 1959		13.0	136.0	Terminal

TABLE XIV

Follow-up

Case 11, Acute Tubular Necrosis, Transfusion Reaction

General Supportive Care: Fluid Balance, Testosterone

Date	NPN mg./100 mls.	Free-17-OHCS µg./100 mls. Plasma	17-OHCS-Gluc. µg./100 mls. Plasma	Comment
Feb. 26, 1959	195	64.5	930.0	
Mar. 1, 1959	195	18.4	875.0	Before dialysis
Mar. 1, 1959	171	20.7	799.0	After dialysis
Mar. 5, 1959		20.4	463.0	Diuresing
Mar. 7, 1959	307	9.7	360.0	Diuresing
Mar. 9, 1959	348			
Expired				

TABLE XV

Follow-up

Case 12, (Female, 12 yrs.) Acute Glomerulonephritis

General Supportive Care: Fluid Balance, Streptomycin, PAS, Testosterone, Digitoxin and Hydrocortisone Throughout

Date	NPN mg./100 mls.	Free-17-OHCS µg./100 mls. Plasma	17-OHCS-Gluc. µg./100 mls. Plasma	Comment
Dec. 29, 1958	187			
Jan. 2, 1959	279			
Jan. 3, 1959	276	78.4	774.0	
Jan. 6, 1959	328			
Jan. 8, 1959	368	50.3	694.0	Before dialysis
Jan. 9, 1959	106	57.5	614.0	After dialysis

rose only very slightly during the period. The 17-hydroxy-corticosteroid-glucuronides fell 10 to 30% during dialyses as did the NPN.

4) Evaluation of the Nelson-Samuels Method.

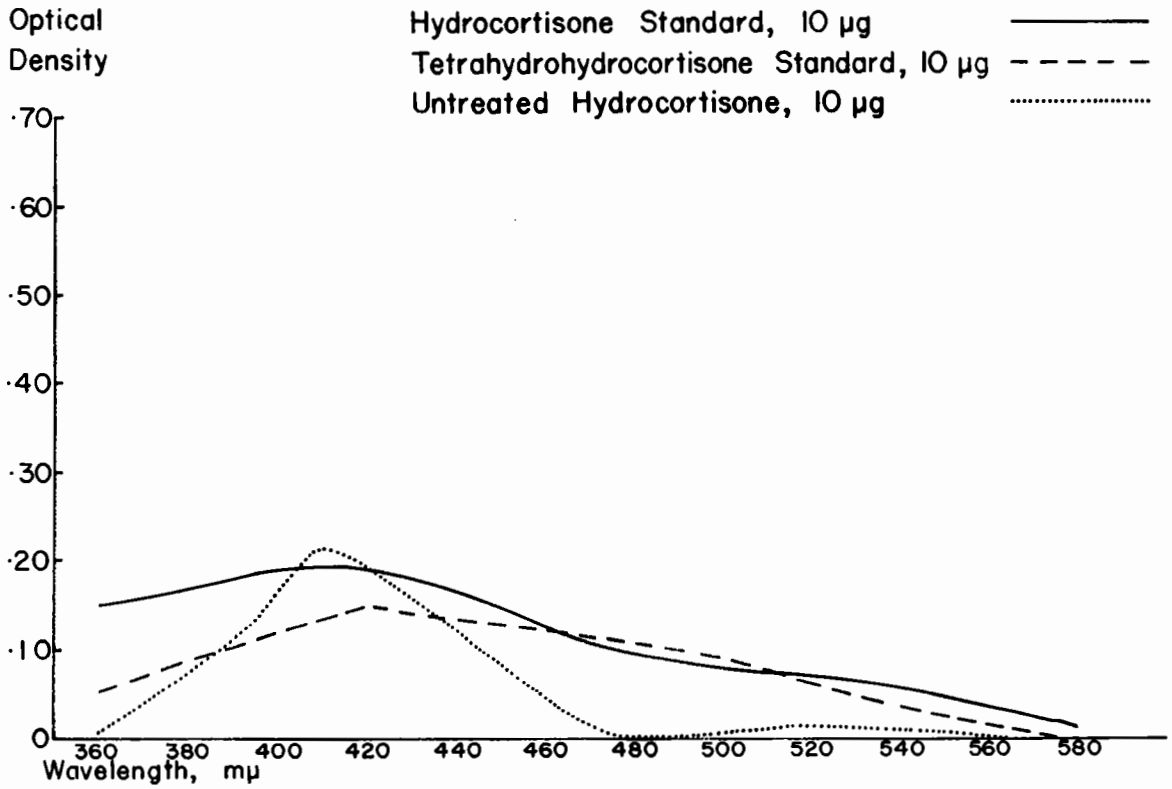
a) Precision and Accuracy of the Method: Recovery experiments have been performed on water, plasma and urine with added free-hydrocortisone. Eighteen recoveries of 5 and 10 µg. of hydrocortisone added to water have revealed a mean recovery of 98% with a standard deviation of ±10% of the mean. Twenty-eight recoveries of 10 µg. of hydrocortisone added to plasma have yielded a mean recovery of 104% with a standard deviation of ±18%. Nine recoveries of hydrocortisone, ranging in amounts from 0.5 to 10.0 µg. per sample and added to urine gave a mean of 104% with a standard deviation of ±15%. Recoveries of amounts below 0.5 µg. per 20 mls. of sample were subject to greater variation. In 35 duplicate determinations on urine samples with a mean sample value of 8 µg. per sample (range 0.6 to 28.0 µg. per sample) the mean of the difference between samples was 0.96 µg. or 12% of the mean.

b) The Purity of the Final Eluate: An ultra-

violet absorption spectra on the dried residue of the final eluate taken up in methyl alcohol of 10 µg. standards of hydrocortisone and tetrahydrocortisone showed a peak absorption at 240-242 mµ. The ultraviolet spectra for the final eluate of samples of urine, plasma and dialyses were not characteristic because of interfering peaks in the neighborhood of 270-280 mµ. The visible light spectra of the phenylhydrazine chromagens of the standards of hydrocortisone and tetrahydrocortisone and the samples of urine, plasma and dialysate gave characteristic absorption peaks at or close to 410 mµ. Figures II and III. None of these peaks, however, were as sharp as that of the untreated 10 µg. of hydrocortisone. The plasma peak is the least characteristic; this is probably due to impurities. The sulfuric acid absorption in the visible light range for these standards and samples was low and constant with a range in optical density of 0.010 to 0.050. Spectra of the phenylhydrazine chromagens on a series of eluates of methyl alcohol and chloroform, which had been passed through a column of Florisil containing 10 µg. of hydrocortisone, showed that there was peaking at 410 mµ. only in the eluate made up of 25%

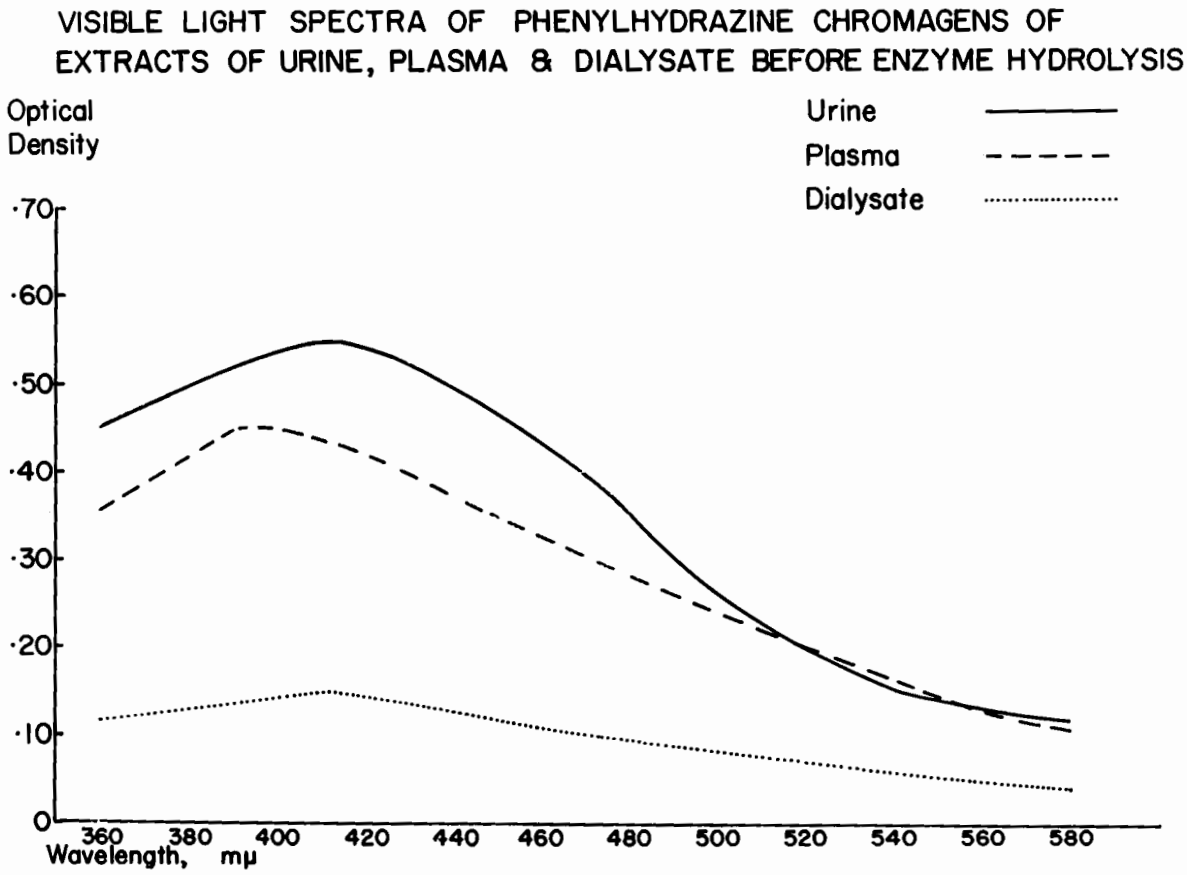
FIGURE II

VISIBLE LIGHT SPECTRA OF PHENYLHYDRAZINE CHROMAGENS OF HYDROCORTISONE AND TETRAHYDROCORTISONE STANDARDS



In the spectra shown above the optical density expressed as degree of absorption of visible light is plotted along the ordinate while the wave length, in mµ., is plotted along the abscissa.

FIGURE III



For explanation see Figure II.

methyl alcohol and 75% chloroform. Figure IV.

5) Evaluation of the Enzyme Hydrolysis:

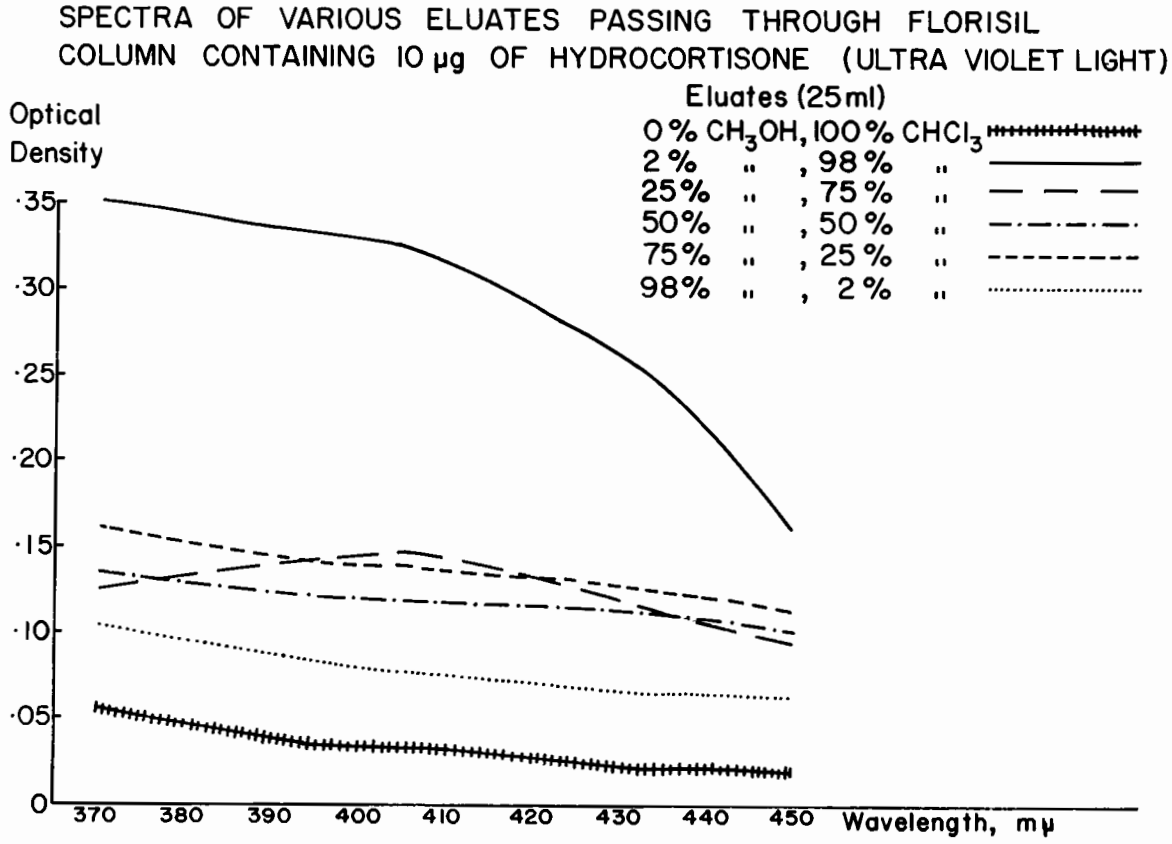
Six recoveries of tetrahydrohydrocortisone-monoglucuronide added to urine in the range of 3.6 to 21.9 µg. per sample revealed a mean recovery of 98% with a standard deviation of ±2%. Duplicate determinations of total (free plus glucuronide) 17-hydroxycorticosteroids on 24 urine samples with a range of 0.99 to 17.0 µg. per sample gave a mean difference between samples of 0.86 µg. per sample or 12% of the mean sample value of 7 µg. Following the extraction of both the free-17-hydroxycorticosteroids and the 17-hydroxycorticosteroid-glucuronides the residual aqueous phases of nine individual samples were subjected to pepsin digestion at pH 1.5 for 24 hours. In 5 out of the nine samples significant chromagenic material was released. Table XVI. Time did not permit this material to be further characterized. When a standard β-glucuronidase incubation was re-employed significant chromagenic material was found in only 1 out of 9 samples. Table XVI. On pooled plasma subsequent to the extraction of free-17-hydroxycorticosteroids and 17-hydroxycorticosteroid-glucuronides, no significant chromagenic material was released after 24 hours of incubation at pH 1.5, with

pepsin at pH 1.5, at pH 4.5, with β-glucuronidase at pH 4.5, at pH 8 and with trypsin at pH 8. Table XVII. These determinations were done in duplicate. Visible light spectra on the phenylhydrazine chromagens released from urine and dialysate following β-glucuronidase incubation showed the characteristic peak at 410 mμ. Figure V. Their sulfuric acid background gave a low and constant absorption. The absorption spectrum for the plasma following β-glucuronidase incubation is not characteristic and the sulfuric acid background had an optical density in the neighborhood of 0.200 mμ.

6) Evaluation of the Accuracy of Dialysis.

Three recovery experiments were performed in which 5, 10 and 15 μg. of hydrocortisone per sample were added to plasma before dialysis. The total amounts recovered from the plasma and the dialysate were respectively 62%, 100% and 122%. The mean recovery was 95% with a standard deviation of 23%. Cellophane, when extracted with methyl alcohol, gives a variable pale rust color with both the sulfuric acid blank reagent and the phenylhydrazine reagent. When the cellophane was extracted following dialysis, the optical density of the sulfuric

FIGURE IV



For explanation see Figure II. Note that ultraviolet light was used in this study because a tungsten bulb was not available at the time the study was performed. At these wave lengths it should not introduce significant variation.

TABLE XVI

Evaluation of Completeness of β-Glucuronidase Hydrolysis on Plasma

Samples of 20 mls.

Phenylhydrazine Chromagens

Case	Extraction #1 µg./ml.	Extraction #2 After β-Glucuronidase µg./ml.	Extraction #3 After Pepsin* µg./ml.	Extraction #4, After β-Glucuronidase µg./ml.
10 F	.69	1.83	.31	.03
10 F	.54	1.44	.35	.02
10 F	1.05	1.45	.20	.01
4 F	.44	.53	0	0
4 F	.38	.23	.42	0
4 F	.38	.34	0	0
4 F	.10	.27	.23	.40
4 F	.27	.14	0	0
4 F	.25	.14	0	0

*100 mg./sample

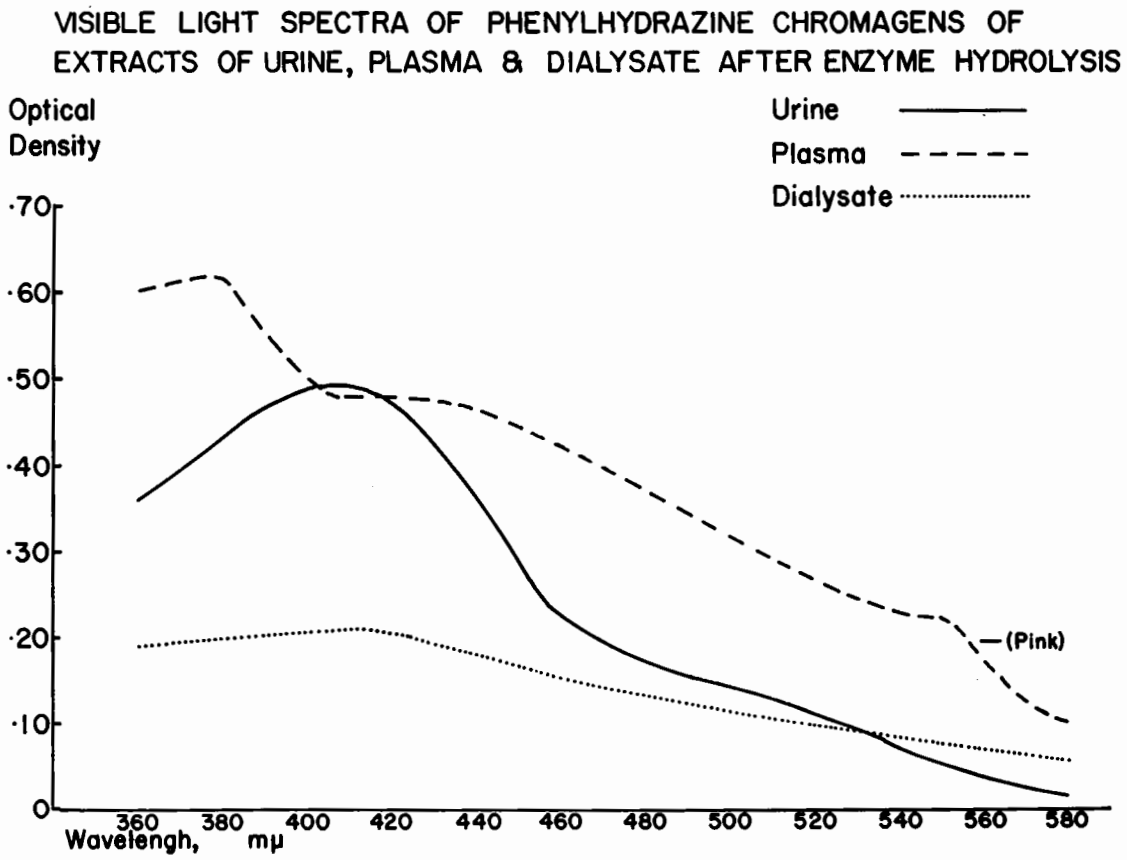
TABLE XVII

Evaluation of Completeness of β-Glucuronidase Hydrolysis on 15 ml. Aliquots of Pooled Plasma

Extraction	Incubation	Phenylhydrazine Chromagens, μg./ml. (Hydrocortisone Equivalents)
No. 1	None	.316
No. 2	β-Glucuronidase	.515
No. 3	pH 1.5	0
No. 3	Pepsin, pH 1.5*	0
No. 3	pH 4.5	0
No. 3	β-Glucuronidase, pH 4.5	0
No. 3	pH 8	0
No. 3	Trypsin, pH 8*	.04

*300 μg./sample

FIGURE V



See Figure IV for explanation.

acid chromagens and the phenylhydrazine chromagens were of the same order.

7) Evaluation of the Effect of Freezing and Storage upon the 17-Hydroxycorticosteroid Content of Samples and Standards.

If standards made up of 5 or 10 µg. of hydrocortisone in water were frozen and stored along with the samples, it was noted that their optical density progressively decreased over a three-month period. Serial recovery experiments on water confirmed this. Tables XVIII and XIX. No hydrocortisone could be extracted from the glass of the tubes in which the water was frozen. Table XIX. There was no loss of recoverable chromagenic material from plasma over a three-month period. Table XX. Likewise, recoveries, over a three-month period on dried extracts of plasma, which was stored at 4°C., and on frozen plasma to which hydrocortisone was added freshly upon thawing were within normal limits. Reproductibility experiments on urine showed that the recoverability of the free chromagenic material started to fall off within 2 months following freezing. Table XXI. There was no demonstrable decrease in the recoverability of total chromagens over a six-month period. Table XXI.

TABLE XVIII

Recoveries of Hydrocortisone Added to Water and Frozen

Hydrocortisone Added to Water µg./20 mls.	Duration of Freezing	Recovery µg./Sample	%
10	None	9.3	93
10	None	10.9	109
10	None	9.6	96
10	Overnight	7.9	79
10	Overnight	8.4	84
10	Overnight	11.0	110
10	3 months	5.25	53
10	3 months	4.64	46
10	3 months	5.06	51
5	8 months	0	0
5	8 months	0	0

TABLE XIX

Recoveries of Hydrocortisone Added to Water and Frozen

Hydrocortisone Added to Water µg./20 mls.	Duration of Freezing	Recoveries µg./Sample	%	From Glass
10	None	10.4	104	0
10	None	9.5	95	0
10	None	10.1	101	0
10	None	11.4	114	0
10	None	8.4	84	0
10	None	10.5	105	0
10	None	8.7	87	0
10	None	9.1	91	0
10	None	9.4	94	0
10	Overnight	11.1	111	0
10	Overnight	10.6	106	0
10	Overnight	10.2	102	0
10	3 months	5.9	59	0
5	3 months	3.3	66	0
5	3 months	2.6	52	0

TABLE XX

Recoveries of Hydrocortisone Added to Plasma and Frozen

Hydrocortisone Added to Plasma µg./20 mls.	Duration of Freezing	Recovery µg./Sample	%
10	None	8.6	86
10	None	11.4	114
10	7 Days	6.3	63
10	7 Days	8.8	88
10	1 Month	10.2	102
10	1 Month	11.5	115
10	2 Months	9.0	90
10	2 Months	9.2	92
10	3 Months	11.8	118
10	3 Months	11.8	118

TABLE XXI

Reproducibility of Results on Frozen Urine

Duration of Freezing	Free-17-OHCS µg./ml. (20 ml. Aliquot)	% of Control	Total 17-OHCS µg./ml. (5 ml. Aliquot)	% of Control
None (Control)	.430 (Mean of 6)	100	3.642 (Mean of 4)	100
2 Months	.361	84	3.81	104
2 Months	.323	75	3.92	108
3 Months	.289	67	3.89	107
3 Months	.216	50	4.33	119
6 Months	.286	67	4.08	112
6 Months	.233	54		
7 Months	.330	77		
7 Months	.208	48		

8) Preliminary Renal Clearance Studies.

a) Protocol: During routine ACTH tests on 3 normal males renal clearance studies were performed at the times of blood sampling; 0, 4, and 48 hours. The clearance periods were of two hours' duration and the blood samples were drawn at or close to the midpoint minus 6 minutes. The "0" hour dose of ACTH was delayed 1 hour so that it was actually given at 9 a.m. on day 3. An oral water load was given to the subjects; this was started about 30 minutes prior to the beginning of the clearance period and continued during the first hour. The subjects voided at the start and the end of each period. An attempt was made to completely empty the bladder. All urine collected between the start and the end of the period was saved and pooled. The creatinine content of the urine and undialyzed plasma was determined. A 20 ml. aliquot of plasma was dialyzed. The free-17-hydroxycorticosteroid and 17-hydroxycorticosteroid-glucuronide content of the urine, dialysate and plasma was determined.

b) Experimental Subjects: The subjects were 3 normal, healthy males (W.M., W.B. and J.B.), two medical

students and one graduate student ages 24, 23 and 34.

c) Calculation of Renal Clearances: The renal clearance values for creatinine and the nonprotein-bound moieties of both the free-17-hydroxycorticosteroids and the 17-hydroxycorticosteroid-glucuronides of the plasma were calculated according to the formula set forth by Smith (275):

$$\text{Renal Clearance} = \frac{U \times V}{P}$$

Where:

U = The concentration of the substance per ml. urine.

V = The minute volume of the urine expressed in ml.

P = The concentration of the substance per ml. of plasma.

In these preliminary studies, since no catheters were used to collect urine, no attempt was made to calculate the renal tubular reabsorption or renal tubular secretion of these hormone fractions. However, by expressing the hormone clearances in ratios in which the hormone clearance is divided by the creatinine clearance (an estimate of the glomerular filtration rate) tubular secretion is implied, if the ratio exceeds 1.0, and

tubular reabsorption, if the ratio is less than 1.0. Any errors in the urine volume would cancel out by using such a ratio as shown below:

$$\frac{U_1 \times V}{P_1} \Big/ \frac{U_2 \times V}{P_2} = \frac{U_1}{P_1} \Big/ \frac{U_2}{P_2}$$

Yet, without catheters, the method cannot be accurate enough to calculate tubular activity in terms of μg./min.

d) Results: The results as to the Porter-Silber chromagens of the plasma and urine specimens are listed in Tables XXII, XXIV and XXVI for W.M., W.B. and J.B., respectively. W.B.'s 48-hour plasma dialysate was lost through a laboratory accident. All of J.B.'s 4-hour specimens (urine, plasma and dialysate) were contaminated with dust and gave high background readings on the spectrophotometer. At the particular time that these determinations were being carried out, heavy construction work was being undertaken in the immediate laboratory area and the amount of atmospheric dust was quite high.

The calculated renal clearance data in these cases are listed in the same sequence in Tables XXIII, XXV, and XXVII. The consistent finding is that the clearances for the nonprotein-bound (dialysable) moiety for both the free-17-hydroxycorticosteroids and the

17-hydroxycorticosteroid-glucuronides increase following the administration of ACTH.

The renal clearance values in the case of W.M. for the nonprotein-bound free-17-hydroxycorticosteroids at 0, 4 and 48 hours were 27.3, 55.8 and 125.5 mls./min. while those for the nonprotein-bound glucuronides were 52.0, 137.5 and 388.2 mls./min. (Table XXIII). The ratio with the creatinine clearance at 48 hours exceeded 1.0 for both the free and glucuronide fraction; these were 1.2 and 3.8, respectively. As far as the glucuronide fraction is concerned, this is significant and implies renal tubular secretion; however, because of the limits of the methods, the same cannot be inferred from the ratio of 1.2 for the free fraction. These changes in the renal clearances of the hormone moieties were observed despite no appreciable increase in the nonprotein-bound free moiety of the plasma or the nonprotein-bound or protein-bound moieties of the glucuronide fraction of the plasma. The total free-17-hydroxycorticosteroids of the plasma increased slightly from 0.134 to 0.180 µg./ml.

As to W.B., his renal clearance values for the free fraction rose from a 0-hour level of 3.7 mls./min. to

41.8 mls./min. at 4 hours; a 48-hour value for the free fraction is not available (Table XXV). The clearances for the glucuronide fraction were 12.2, 105.2 and 605.0 mls./min. at 0, 4 and 48 hours. The only hormone clearance/creatinine clearance ratio to exceed 1.0 was that for the glucuronides at 48 hours; it was 4.2 which is a value that definitely indicates tubular secretion. In this study, the control plasma corticoid value at 0 hour is abnormally high at 0.37 µg./ml.; of this 0.33 µg./ml. was nonprotein bound which is a very high percentage for the amount of nonprotein-bound hormone; unfortunately, the plasma protein binding affinities were not studied. Apparently the subject had been under some undefined stress preceding the study because the spectrophotometric readings would seem to be dependable. The plasma glucuronides were also high and this would tend to confirm the impression that he had been previously stressed. The free-17-hydroxycorticosteroids of the plasma, both nonprotein-bound and total moieties, showed a decrease from the control levels at 4 hours. The nonprotein-bound and total plasma glucuronides also were decreased at 4 and 48 hours.

The renal clearances of the nonprotein-bound hormone moieties, in J.B., likewise showed an increase after ACTH administration (Table XXVII). Those of the free fraction rose from a 0-hour value of 7.5 mls./min. to a 48-hour level of 70.8 mls./min. while those of the glucuronides went from 94.4 to 126.5 mls./min. over the same period. None of the ratios with the creatinine clearance surpassed 1.0. During this study, both the nonprotein-bound moiety and the total fraction of the free-17-hydroxycorticosteroids of the plasma showed an increase at 48 hours over the 0-hour levels. Nonprotein-bound glucuronides of the plasma increased at the same time; no protein-bound glucuronides were demonstrable in this case at either 0 or 48 hours.

The results of these preliminary clearance studies showed an increase in the renal clearances of the nonprotein-bound moiety of both the free and glucuronide fractions of the 17-hydroxycorticosteroids in association with the administration of ACTH. In 2 out of 3 of these, the glucuronide clearances rose to surpass the creatinine clearance by significant margins which would imply renal tubular secretion of this fraction at

TABLE XXII

Urinary and Plasma 17-Hydroxycorticosteroids Before and During ACTH Stimulation

Clearance Period	Urinary 17-OHCS		Plasma 17-OHCS-			
	Free µg./ml.	Glucuronides µg./ml.	Nonprotein-bound Free (µg./ml.)	Protein-bound Free (µg./ml.)	Nonprotein-bound Glucuronides (µg./ml.)	Protein-bound Glucuronides (µg./ml.)
			(Allen Correction)			
$U_{0\ Hr.}$	1.01	16.60	.044	.090	.380	0
$U_{4\ Hr.}$	1.95	19.64	.050	.130	.205	.165
$U_{48\ Hr.}$	1.05	15.60	.050	.130	.240	.034
			(Blank Correction)			
$U_{0\ Hr.}$ (Repeat)	.318	2.13	.080	.212	.803	0

Subject: W.M., 24, male.

TABLE XXIII

Renal Clearances of 17-Hydroxycorticosteroids Before and During ACTH Stimulation

Clearance Period	Urine min. vol. ml.	Clearances: Creatinine mls./min. (C_{CR})	Clearances: Nonprotein-bound Free 17-OHCS mls./min. (C_{S-D})	Clearances: Nonprotein-bound 17-OHCS-Glucuronides mls./min. (C_{SG-D})	Clearance Ratios: C_{S-D}/C_{CR} [1]	Clearance Ratios: C_{SG-D}/C_{CR} [2]
			(Allen Correction)			
U_{0} Hr.	1.19	94.9	27.3	52.0	.3	.6
U_{4} Hr.	1.43	138.6	55.8	137.5	.4	1.0
U_{48} Hr.	5.97	101.8	125.5	388.2	1.2	3.8
			(Blank Correction)			
U_{0} Hr.	5.8	165	23.0	15.4	.14	.09

Subject: W.M., 24, male.

1. $\frac{\text{Nonprotein-bound Free-17-OHCS Clearance}}{\text{Creatinine Clearance}}$

2. $\frac{\text{Nonprotein-bound 17-OHCS-Glucuronide Clearance}}{\text{Creatinine Clearance}}$

TABLE XXIV

Urinary and Plasma 17-Hydroxycorticosteroids Before and During ACTH Stimulation

Clearance Period	Urinary 17-OHCS		Plasma 17-OHCS			
	Free µg./ml.	Glucuronides µg./ml.	Nonprotein-bound Free (µg./ml.)	Protein-bound Free (µg./ml.)	Nonprotein-bound Glucuronides (µg./ml.)	Protein-bound Glucuronides (µg./ml.)
			(Allen Correction)			
$U_{0\ Hr.}$	.363	1.05	.330	.040	.288	.215
$U_{4\ Hr.}$	.833	2.85	.168	.048	.228	.113
$U_{48\ Hr.}$	1.03	7.2	----[1]	.238[2]	.101	.273
			(Blank Correction)			
$U_{0\ Hr.}$ (Repeat)	.246	1.6	.190	.130	.220	.260

Subject: W.B., 22, male.

1. Specimen lost

2. Uncorrected

TABLE XXV

Renal Clearances of 17-Hydroxycorticosteroids Before and During ACTH Stimulation

Clearance Period	Urine min. vol. mls.	Clearances			Clearance Ratios[1]	
		Creatinine mls./min.	Nonprotein-bound Free 17-OHCS mls./min.	Nonprotein-bound 17-OHCS-Glucuronides mls./min.	C_{S-D}/C_{CR}	C_{SG-D}/C_{CR}
		(C_{CR})	(C_{S-D})	(C_{SG-D})		
			(Allen Correction)			
U_{0} Hr.	3.35	110	3.7	12.2	.03	.11
U_{4} Hr.	8.42	133	41.8	105.2	.32	.8
U_{48} Hr.	8.50	143	---	605.0	---	4.2
			(Blank Correction)			
U_{0} Hr. (Repeat)	5.50	116	7.12	40.2	.06	.34

Subject: W.B., 22, male.

1. For explanation see Table XXIII.

TABLE XXVI

Urinary and Plasma 17-Hydroxycorticosteroids Before and During ACTH Stimulation

Clearance Period	Urinary 17-OHCS		Plasma 17-OHCS			
	Free µg./ml.	Glucuronides µg./ml.	Nonprotein-bound Free (µg./ml.)	Protein-bound Free (µg./ml.)	Nonprotein-bound Glucuronides µg./ml.	Protein-bound Glucuronides µg./ml.
			(Allen Correction)			
$U_{0\ Hr.}$	.068	1.96	.053	.070	.122	0
$U_{4\ Hr.}$	----[1]	----[1]	----[1]	----[1]	----[1]	----[1]
$U_{48\ Hr.}$	1.43	10.3	.128	.449	.517	0
			(Blank Correction)			
$U_{0\ Hr.}$ (Repeat)	.139	1.39	.015	.230	.106	0

Subject: J.B., 34, male

1. High background.

TABLE XXVII

Renal Clearances of 17-Hydroxycorticosteroids Before and During ACTH Stimulation

Clearance Period	Urine min. vol. mls.	Clearances: Creatinine mls./min. (C_{CR})	Clearances: Nonprotein-bound Free-17-OHCS mls./min. (C_{S-D})	Clearances: Nonprotein-bound 17-OHCS-Glucuronides mls./min. (C_{SG-D})	Clearance Ratios[1]: C_{S-D}/C_{CR}	Clearance Ratios[1]: C_{SG-D}/C_{CR}
			(Allen Correction)			
U_{0} Hr.	5.86	113.5	7.5	94.4	.07	.83
U_{4} Hr.	8.03	114.2	---	---	---	---
U_{48} Hr.	6.34	135.4	70.8	126.5	.52	.94
			(Blank Correction)			
U_{0} Hr. (Repeat)	7.67	148.8	70.6	100.6	.47	.68

Subject: J.B., 34, male.

1. For explanation, see Table XXIII.

those times. The associated glucuronide plasma fractions showed an increase in only 1 out of these 3 subjects during these studies.

9) Renal Clearance Studies During ACTH and Cortisol-PO_4 Disappearance.

a) Protocol: The same protocol as described in Experiments and Results, Part 8 "a," was employed for the studies before and during ACTH stimulation. The clearance periods during cortisol phosphate disappearance studies were of 60 minutes' duration. The blood samples were not necessarily drawn near the midpoint of the clearance periods during these latter studies; instead, serial values were plotted on a semi-log ordinate against time on the abscissa. The values at the clearance period's midpoint minus 6 minutes were interpolated from the graph. Cortisol phosphate was administered intravenously, 1 mg./Kg. of body weight, over a 20-minute period between a control clearance period and the U_1 clearance period. An indwelling catheter was used for all urine collections in B.G. A small amount of distilled water was measured and used to wash out the bladder and catheter at the end of each clearance period. W.B. voided but carefully tried to completely empty the bladder each time.

b) Experimental Subjects: The subject B.G. was a 19-year-old female, 80 kg. in weight and 165 cm. in height, with moon faces, truncal obesity, hirsutism and mild hypertension. Her plasma and urinary 17-hydroxycorticosteroids were elevated on repeated occasions. Following the studies shown below, she was found to have what appeared to be unilateral hypertrophy of the left adrenal cortex at adrenalectomy. W.B. was a 22-year-old normal, male medical student, 70 kg. in weight and 175 cm. in height.

c) Calculations: Renal clearances were calculated as described in part 8 "c" of Experiments and Results. The determined plasma values of the various moieties were used to construct the semi-log line graphs shown in Figures VI and VIII. From these lines, the estimated plasma levels of the various moieties at the appropriate times were interpolated.

The net tubular activity, which was not determined in the preceding preliminary studies, was expressed in terms of μg./min. and was calculated according to the method of Smith (275):

$$T_X = (P_X \cdot C_{CR}) - (U_X \cdot V)$$

Where:

T_X = The renal tubular activity of substance X in μg. per minute.

P_X = The concentration of substance X per ml. of plasma.

C_{CR} = The creatinine clearance in mls. per minute.

U_X = The concentration of substance X per ml. of urine.

V = The minute volume of urine in ml.

It is evident that $U_X \cdot V$ is the amount present in the urine after tubular activity and that $P_X \cdot C_{CR}$ is the amount in the filtered load at the glomerular membrane prior to any tubular activity. The difference between these two values represents only the net tubular activity. If the algebraic sum of the right hand side of the equations is plus, then it stands to reason that renal tubular reabsorption has taken place, for the theoretical concentration in the filtered load would have exceeded the amount of hormone recovered from the urine.

The amounts of protein-bound free-17-hydroxycorticosteroids which should be present in the total plasma volume and the amount of nonprotein-bound free-17-hydroxycorticosteroid which should be present in the

total plasma volume, extracellular fluid volume or the total body water volume were calculated by employing the appropriate percentages of body weight as set forth by Gamble (276): 5 percent for plasma volume, 20 percent for extracellular fluid volume, and 70 percent for total body water volume. Variations in the percentages as determined by other authors are not significant within the limits of error which were obtained in the determination of these nonprotein-bound and protein-bound fractions.

d) Results: The results of the determination of the Porter-Silber chromagens in the plasma and urine specimens during the cortisol-phosphate disappearance studies are shown in Tables XXVIIIa and XXXIa for B.G. and W.B., respectively. Those determined for 0, 4 and 48 hours of ACTH administration are listed in Tables XXVIIIb and XXXIb together with the interpolated plasma values for the disappearance studies; it was these interpolated values which were used in the calculation of the renal clearance data for the disappearance studies. The semi-log disappearance curve for B.G. is seen in Figure VI and in Figure VIII for W.B. In Tables XXVIIIc and XXXIc are listed the estimated total free hormone

Subheading to FIGURE VI:

In the above graph, the term "dialysable" is used to denote the nonprotein-bound moiety and "non-dialysable" is used to denote the protein-bound moiety. None of the 17-hydroxycorticosteroid glucuronides were found to be protein bound (nondialysable). These protein-bound and nonprotein-bound moieties of the free and glucuronide fractions of the 17-hydroxycorticosteroids present in the plasma are plotted semi-logarithmically against time on the abscissa.

FIGURE VI

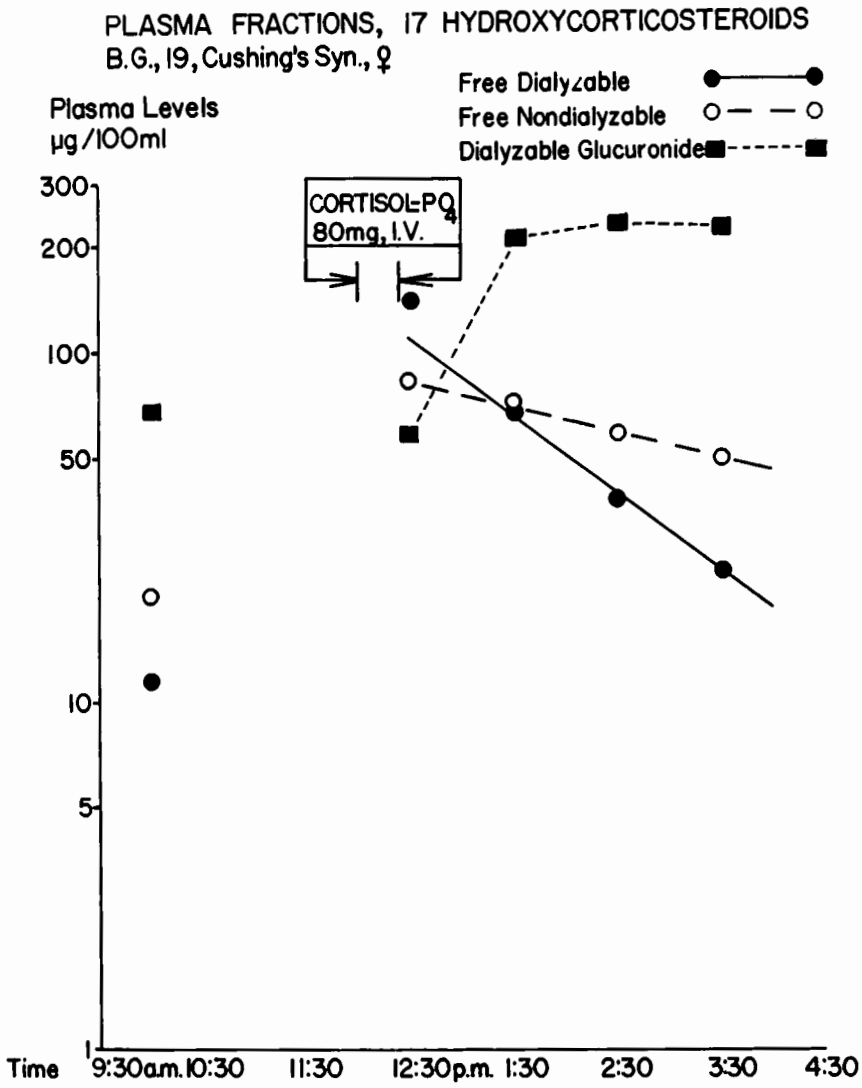

PLASMA FRACTIONS, 17 HYDROXYCORTICOSTEROIDS
B.G., 19, Cushing's Syn., ♀
Free Dialyzable
Free Nondialyzable
Dialyzable Glucuronide
Plasma Levels
µg/100ml
CORTISOL-PO4
80mg, I.V.
300
200
100
50
10
5
1
Time
9:30a.m.
10:30
11:30
12:30p.m.
1:30
2:30
3:30
4:30

TABLE XXVIII a

Actual Plasma 17-Hydroxycorticosteroid Values During Cortisol-PO_4 Disappearance

	Plasma 17-OHCS			
Time	Nonprotein-bound Free (µg./ml.)	Protein-bound Free (µg./ml.)	Nonprotein-bound Glucuronides µg./ml.	Protein-bound Glucuronides µg./ml.
10:00 a.m.	.111	.196	.678	0.0
12:30 p.m.	1.416	.832	.577	0.0
1:30	.658	.681	2.079	0.0
2:30	.379	.584	2.280	0.0
3:30	.235	.489	2.220	0.0

Subject: B.G., female, 19, Cushing's Syndrome.

TABLE XXVIII b

Urinary and Plasma 17-Hydroxycorticosteroids During ACTH Stimulation and Cortisol-PO_4 Disappearance

Clearance Period	Urinary 17-OHCS		Plasma 17-OHCS			
	Free µg./ml.	Glucuronides µg./ml.	Nonprotein-bound Free (µg./ml.)	Protein-bound Free (µg./ml.)	Nonprotein-bound Glucuronides µg./ml.	Protein-bound Glucuronides µg./ml.
			(ACTH Stimulation)			
$U_{0\ Hr.}$	.280	4.160	.058	.567	1.825	0.0
$U_{4\ Hr.}$	1.370	9.660	.174	.364	1.538	0.0
$U_{48\ Hr.}$	2.330	10.440	.170	.875	1.330	.119
			(Cortisol-PO_4 Disappearance)[1]			
$U_{control}$	.127	1.807	.111	.196	.678	0.0
U_1	2.585	14.120	.960	.760	.920	0.0
U_2	4.403	38.710	.530	.660	2.15	0.0
U_3	2.026	16.730	.290	.570	2.25	0.0
U_4	3.790	49.900	.157	.480	2.15	0.0

Subject: B.G., 19, female, Cushing's Syndrome

1. Plasma values interpolated from semi-log line graph, Figure VI.

TABLE XXVIII c

Estimated 17-Hydroxycorticosteroid Content of the Fluid Compartments of the Body in B.G. During Cortisol-Phosphate Disappearance Studies

Moiety and Fluid Compartment	mg./Fluid Vol.
Nonprotein-bound free-17-OHCS per plasma volume	4.56
Nonprotein-bound free-17-OHCS per E.C.F.[1] volume	18.23
Nonprotein-bound free-17-OHCS per T.B.W.[2] volume	63.90
Protein-bound free-17-OHCS per plasma volume	2.72
	mg./dose
Cortisol-Phosphate Administered	80.0

1. E.C.F.: Extracellular Fluid

2. T.B.W.: Total Body Water

Plasma volume has been calculated as being equal to 5% of body weight, E.C.F. volume as being equal to 20% and T.B.W. volume as being equal to 70%, after the values suggested by Gamble (276).

TABLE XXIX

Renal Clearances of 17-Hydroxycorticosteroids During ACTH Stimulation and Cortisol-PO_4 Disappearance

Clearance Period	Urine min. vol. mls.	Clearances: Creatinine mls./min. (C_{CR})	Clearances: Nonprotein-bound Free-17-OHCS mls./min. (C_{SD})	Clearances: Nonprotein-bound 17-OHCS-Glucuronides mls./min. (C_{SGD})	Clearance Ratios: C_{SD}/C_{CR}	Clearance Ratios: C_{SGD}/C_{CR}
			(ACTH Stimulation)			
$U_{0\ Hr.}$	7.02	142	33.8	16.0	.24	.11
$U_{4\ Hr.}$	5.77	166	45.4	38.7	.27	.23
$U_{48\ Hr.}$	9.34	130	128	67.2	.99	.52
			(Cortisol-PO_4 Disappearance)			
$U_{control}$	12.1	151	13.8	32.3	.09	.20
U_1	6.5	206	11.8	99.8	.06	.39
U_2	2.2	129	14.7	39.5	.11	.31
U_3	3.8	164	20.3	28.2	.12	.17
U_4	1.56	135	37.6	35.8	.23	.26

Subject: B.G., 19, female, Cushing's Syndrome.

contents of the various fluid compartments in these two subjects assuming that the protein-bound hormone is present in the plasma volume only and the nonprotein-bound hormone is present either in the extracellular fluid volume or total body water volume, as well as the plasma volume. The calculated renal clearance data is to be found in Tables XXIX and XXXII. The net renal tubular activity is expressed in terms of µg./min. in Tables XXX and XXXIII.

In B.G., the subject with Cushing's syndrome, the renal clearances of both the free nonprotein-bound moiety and the nonprotein-bound glucuronide moiety increased during the administration of ACTH. In neither instance, however, did the hormone clearance/creatinine clearance ratio surpass 1.0; this is confirmed in Table XXX where only net renal tubular reabsorption can be calculated for either moiety. During ACTH administration, the plasma values for the nonprotein-bound and protein-bound free moieties did increase. The nonprotein-bound glucuronide moiety decreased slightly after ACTH was started; insignificant amounts of the glucuronide fraction were demonstrably bound to plasma protein during this study.

TABLE XXX

Net Renal Tubular Activity of the 17-Hydroxycorticosteroids

Period	*Free-17-OHCS µg./min.	*17-OHCS-Glucuronides µg./min.
	(ACTH Stimulation)	
$U_{0\ Hr.}$	+ 6.3[1]	+230.3
$U_{4\ Hr.}$	+ 20.9	+182.65
$U_{48\ Hr.}$	+ 0.28	+ 83.1
	(Cortisol-PO_4 Disappearance)	
$U_{control}$	+ 15.2	+ 24.5
U_1	+180.2	+261.2
U_2	+ 58.8	+193.8
U_3	+ 39.6	+303.5
U_4	+ 15.4	+213.3

Subject: B.G., 19, female, Cushing's Syndrome

1. Plus indicates tubular reabsorption.

* Free 17-OHCS is used to indicate the amount of the free fraction which is theoretically reabsorbed or secreted by the renal tubules; similarly *17-OHCS-Glucuronide for the net renal tubular activity of the glucuronide fraction.

Subheading to FIGURE VII a:

In the above graph the levels of the plasma moieties are shown on the first line; the term "dialysable" is used to signify the nonprotein-bound fraction and "nondialysable," the protein-bound fraction. In the center, the creatinine clearance is plotted from the baseline downward and the hormone clearance (C_{SD}) from the level of the creatinine clearance upward so that in clearance periods where a hormone clearance is greater than the creatinine clearance and, therefore, surpasses the baseline, tubular secretion of the hormone is implied. The lower part of the figure shows, in like fashion, the amount of hormone appearing in the filtered load each minute ($C_{CR}P_{SD}$) balanced against the amount appearing in the bladder urine each minute ($U_{SD}V$).

FIGURE VII "a"

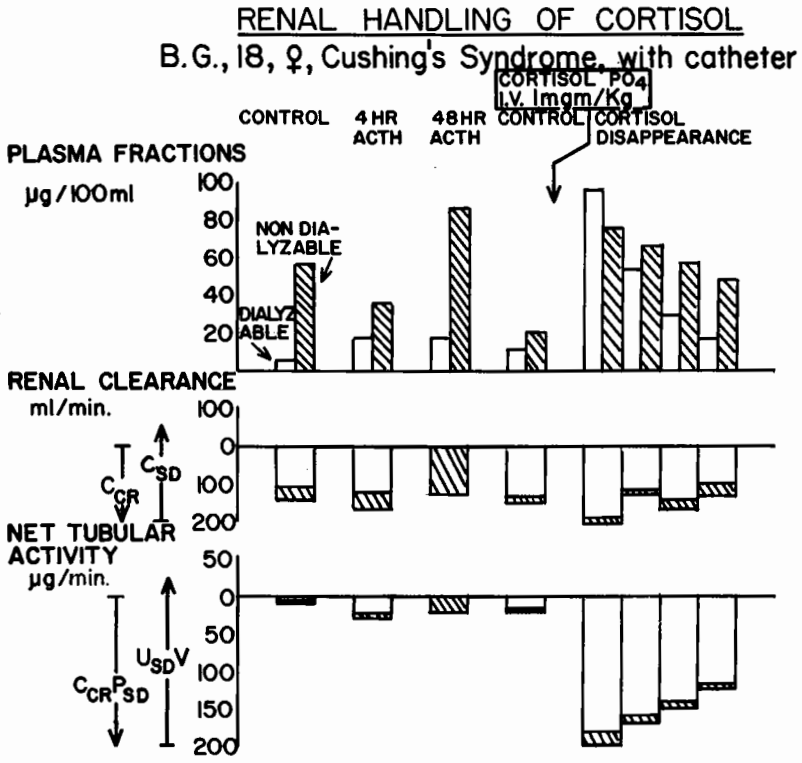
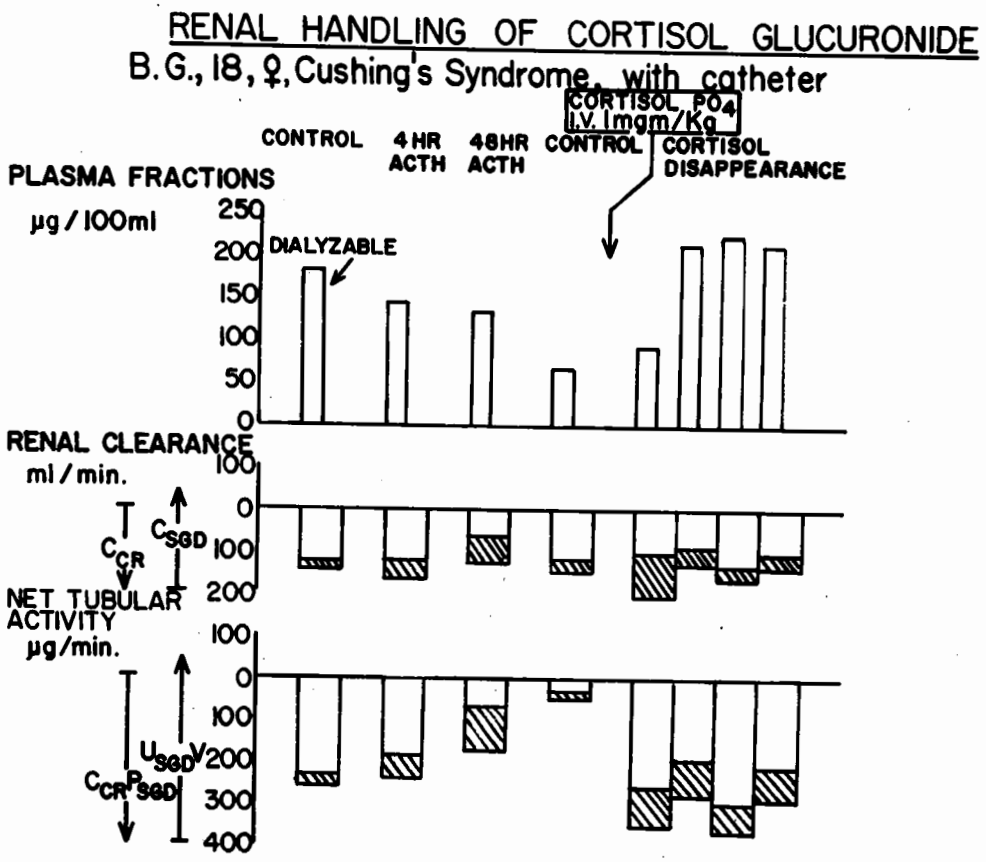

RENAL HANDLING OF CORTISOL
B.G., 18, ♀, Cushing's Syndrome, with catheter
CORTISOL PO4 I.V. Imgm/Kg
CONTROL
4 HR ACTH
48 HR ACTH
CONTROL
CORTISOL DISAPPEARANCE
PLASMA FRACTIONS
µg/100ml
NON DIALYZABLE
DIALYZABLE
RENAL CLEARANCE
ml/min.
NET TUBULAR ACTIVITY
µg/min.

FIGURE VII "b"

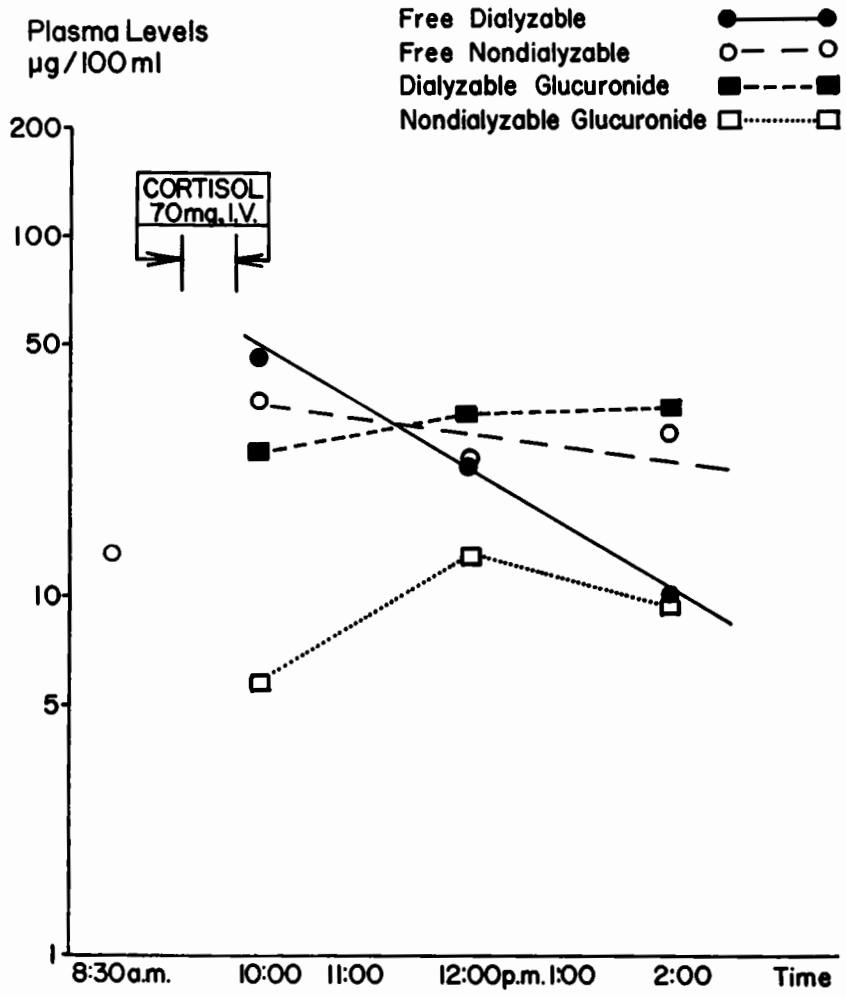


This graph is drawn in a fashion similar to that of FIGURE VII a. C_{SGD} signifies the renal clearance of the glucuronide fraction, $C_{CR}P_{SGD}$ indicates the amount of glucuronide appearing in the filtered load each minute and $U_{SGD}V$ stands for the glucuronide content of a minute volume of urine.

Subheading to FIGURE VIII:

In the above graph, the term "dialysable" is used to denote the nonprotein-bound moieties and "nondialysable" is used to denote the protein-bound moieties. These protein-bound and nonprotein-bound moieties of the free and glucuronide fractions of the 17-hydroxycorticosteroids present in the plasma are plotted semilogarithmically against time on the abscissa.

FIGURE VIII


PLASMA FRACTIONS, 17 HYDROXYCORTICOSTEROIDS
W.B., 22, Normal, ♂
Plasma Levels
µg/100 ml
Free Dialyzable
Free Nondialyzable
Dialyzable Glucuronide
Nondialyzable Glucuronide
200
100
50
10
5
1
CORTISOL
70mg. I.V.
8:30a.m.
10:00
11:00
12:00p.m.
1:00
2:00
Time

During the cortisol-phosphate disappearance study, B.G. showed moderate increases in the renal clearances of both the free nonprotein-bound moiety and the nonprotein-bound glucuronide moiety. In the case of the free fraction, the highest clearance values (37.6 mls/min.) occurred during the final clearance period when the plasma values were returning towards the control levels. The highest glucuronide clearance actually preceded the appearance of the very high plasma glucuronide values. Only renal tubular reabsorption could be calculated for either fraction during this study. The renal clearances and the net tubular activity are graphically charted in Figures VIIa and VIIb. In these, the creatinine clearance (C_{CR}) is plotted from the baseline downward; that of the hormones (C_{SD} for the clearance of the free and C_{SDG} for the clearance of the glucuronides) is plotted from the creatinine clearance level upward. A level higher than that of the baseline would indicate tubular excretion. In the lower part of these graphs, drawn in like fashion, is shown the amount of hormone in the filtered load ($C_{CR}P_{SD}$ for the free fraction and $C_{CR}P_{SGD}$ for the glucuronides) balanced against the amount

appearing in the bladder urine ($U_{SD}V$ and $U_{SGD}V$, respectively); the amounts are expressed in μg./min.

The subject W.B. did not demonstrate any increase in the renal clearance of the free nonprotein-bound moiety during the administration of ACTH, but there was a demonstrable increase of the clearance of the non-protein-bound glucuronide moiety. However, the nonprotein-bound moiety of the free fraction and the protein-bound moiety of the free fraction of the plasma both increased during ACTH whereas the nonprotein-bound glucuronide level actually decreased. Significant amounts of glucuronides in the plasma could not be shown to be protein-bound. Renal tubular excretion of either fraction did not occur while ACTH was given.

During this cortisol-phosphate disappearance study, W.B. showed an increase in the clearance of the free fraction from 74.7 mls./min. to 90.2 mls./min.; the clearance of the free fraction never surpassed the creatinine clearance and the highest clearance levels were not associated with the highest plasma values of either the nonprotein-bound or protein-bound moieties of the free fraction. During the disappearance study, the

clearance of the glucuronides consistently exceeded that of the creatinine, indicating renal tubular secretion of this fraction. The control clearance value is an estimate; since no Porter-Silber chromagens were found in the dialysate, the lower limit of the method, 0.02 µg./ml., was used in the calculations. W.B.'s renal clearance data is graphically shown in Figures IXa and IXb employing the same techniques as in Figures VIIa and VIIb.

The disparity between the disappearance half-times of the nonprotein-bound (dialysable) and the protein-bound (nondialysable) moieties of the free-17-hydroxy-corticosteroids (Figures VI and VIII) suggests that considerable error is incurred in the estimate of the apparent volume of distribution of large doses of infused cortisol at "zero time" by extrapolation of the semi-log regression curves, if the effect of binding by the plasma proteins is not considered. The disappearance half-times for the nonprotein-bound free-17-hydroxycorticosteroids were 84 and 96 minutes for B.G. and W.B., respectively; the half-times for the protein-bound free-17-hydroxy-corticosteroids were 244 and 390 in the same sequence. The regression curve for the protein-bound free-17-

hydroxycorticosteroids in the case of W.B. is a compromise among three points which do not fall exactly along a straight line; even so, despite any error so incurred, the disparity between the disappearance half-times is still apparent and marked. The amount of the nonprotein-bound moiety of the free-17-hydroxycorticosteroids estimated to be contained in either the extracellular fluid volume or the total body water volume, as well as the amount of the protein-bound moiety present in the plasma volume of these two subjects, is listed in Tables XXVIIIc and XXXIc. Peterson et al have shown that the amount of the free-17-hydroxycorticosteroids present in the red cell volume is approximately equal to 55 percent of the total free-17-hydroxycorticosteroid content of the plasma volume (245). This would amount to 4.0 mg. for B.G. and 1.7 mg. for W.B. The amount of the free-17-hydroxycorticosteroids excreted in the urine during the period of the disappearance study should be added to the zero time totals because they have, theoretically, thrown the regression curves off by that amount. It is a small quantity: for B.G., 2.4 mg., and for W.B., 4.1 mg. The amount of cortisol or the degredation products thereof which might appear in the

bile, feces or expired air during the course of these studies is negligible and need not be taken into account (245, 277).

Therefore, if it is assumed that the protein-bound moiety is distributed only into the plasma volume and the nonprotein-bound moiety into the extracellular fluid volume, then, following the above corrections, in the case of B.G. 34 percent of the administered dose can be accounted for or 27.4 out of 80.0 mg.; while in the case of W.B., 22 percent or 15.2 out of 70.0 mg. can be accounted for. If, however, the nonprotein-bound moiety is assumed to be distributed into the total body water volume, then 91 percent or 73.0 out of 80.0 mg. can be accounted for in B.G., and in W.B., 52 percent or 36.2 out of 70.0 mg.

Briefly then, the studies on these two subjects have shown an increase in the renal clearance of the free fraction of the 17-hydroxycorticosteroids in 1 out of the 2 and an increase in the renal clearance of the glucuronide fraction in both subjects during ACTH. In each case, the administration of ACTH was accompanied by increases in the levels of the free-17-hydroxycortico-

TABLE XXXI a

Determined Plasma 17-Hydroxycorticosteroid Values

During Cortisol-PO_4 Disappearance

Plasma 17-OH

Time	Nonprotein-bound Free µg./ml.	Protein-bound Free µg./ml.	Nonprotein-bound Glucuronides µg./ml.	Protein-bound Glucuronides µg./ml.
8:54 a.m.	(< .020)[1]	.132	(< .020)[1]	0.0
10:10	.470	.345	.252	.580
12:06 p.m.	.232	.234	.321	.129
1:57	.098	.286	.338	.096

Subject: W.B., 22, male, normal.

1. When no color developed in the dialysate, 0.02 µg. per ml. (the lower limit of the method) was used for the calculation of the renal clearance of the nonprotein-bound (dialysable) moieties.

TABLE XXXI b

Urinary and Plasma 17-Hydroxycorticosteroids During ACTH Stimulation and Cortisol-PO_4 Disappearance

Clearance Period	Urinary 17-OHCS		Plasma 17-OHCS[1]			
	Free µg./ml.	Glucuronides µg./ml.	Nonprotein-bound Free (µg./ml.)	Protein-bound Free (µg./ml.)	Nonprotein-bound Glucuronides µg./ml.	Protein-bound Glucuronides µg./ml.
			(ACTH Stimulation)			
$U_{0\ Hr.}$	.247	3.500	.030	.052	1.000	0.0
$U_{4\ Hr.}$	.679	2.000	.163	.602	.770	0.0
$U_{48\ Hr.}$	.588	9.130	.248	.214	.673	0.0
			(Cortisol-PO_4 Disappearance)[1]			
$U_{control}$	.277	2.928	(.020)[2]	.132	(.020)[2]	0.0
U_1	4.257	10.073	.420	.330	.265	.265
U_2	2.103	8.397	.280	.295	.300	.300
U_3	5.878	51.822	.185	.260	.310	.310
U_4	1.297	7.171	.125	.235	.330	.330

Subject: W.B., 22, male, normal.

1. Plasma values interpolated from semi-log line graph, Figure VIII.

2. See footnote number 1, Table XXXI a.

TABLE XXXI c

Estimated 17-Hydroxycorticosteroid Content of the Fluid Compartments of the Body in W.B. During Cortisol-Phosphate Disappearance Studies

Moiety and Fluid Compartment	mg./Fluid Vol.
Nonprotein-bound free-17-OHCS per plasma volume	2.10
Nonprotein-bound free-17-OHCS per E.C.F.[1] volume	8.40
Nonprotein-bound free-17-OHCS per T.B.W.[2] volume	29.40
Protein-bound free-17-OHCS per plasma volume	1.02
	mg./dose
Cortisol-Phosphate Administered	70.0

1. E.C.F.: Extracellular Fluid

2. T.B.W.: Total Body Water

Plasma volume has been calculated as being equal to 5% of body weight, E.C.F. volume as being equal to 20% and T.B.W. volume as being equal to 70%, after the values suggested by Gamble (276).

TABLE XXXII

Renal Clearance of 17-Hydroxycorticosteroids During ACTH Stimulation and Cortisol-PO_4 Disappearance

Clearance Period	Urine min. vol. mls.	Clearances: Creatinine mls./min. (C_{CR})	Clearances: Nonprotein-bound Free-17-OHCS mls./min. (C_{SD})	Clearances: Nonprotein-bound 17-OHCS-Glucuronides mls/min. (C_{SDG})	Clearance Ratios: C_{SD}/C_{CR}	Clearance Ratios: C_{SGD}/C_{CR}
			(ACTH Stimulation)			
$U_{0\ Hr.}$	3.46	116	28.5	12.1	.24	.10
$U_{4\ Hr.}$	9.58	128	39.9	24.9	.31	.20
$U_{48\ Hr.}$	6.38	146	15.1	86.5	.10	.60
			(Cortisol-PO_4 Disappearance)			
$U_{control}$	5.4	110	74.7	792.0	.68	7.20
U_1	8.1	122	82.0	306.0	.67	2.50
U_2	8.7	118	66.2	243.0	.57	2.06
U_3	1.63	130	51.8	273.0	.40	2.10
U_4	8.7	135	90.2	188.0	.67	1.40

Subject: W.B., 22, male, normal.

TABLE XXXIII

Net Renal Tubular Activity of the 17-Hydroxycorticosteroids

Period	*Free-17-OHCS µg./min.	*17-OHCS-Glucuronides µg./min.
	(ACTH Stimulation)	
$U_{0\ Hr.}$	+ 2.6[1]	+103.0
$U_{4\ Hr.}$	+14.4	+ 78.8
$U_{48\ Hr.}$	+32.4	+ 39.9
	(Cortisol-PO_4 Disappearance)	
$U_{control}$	+ .7	- 13.6[2]
U_1	+16.7	- 48.9
U_2	+14.6	- 38.5
U_3	+14.5	- 44.1
U_4	+ 5.6	- 17.9

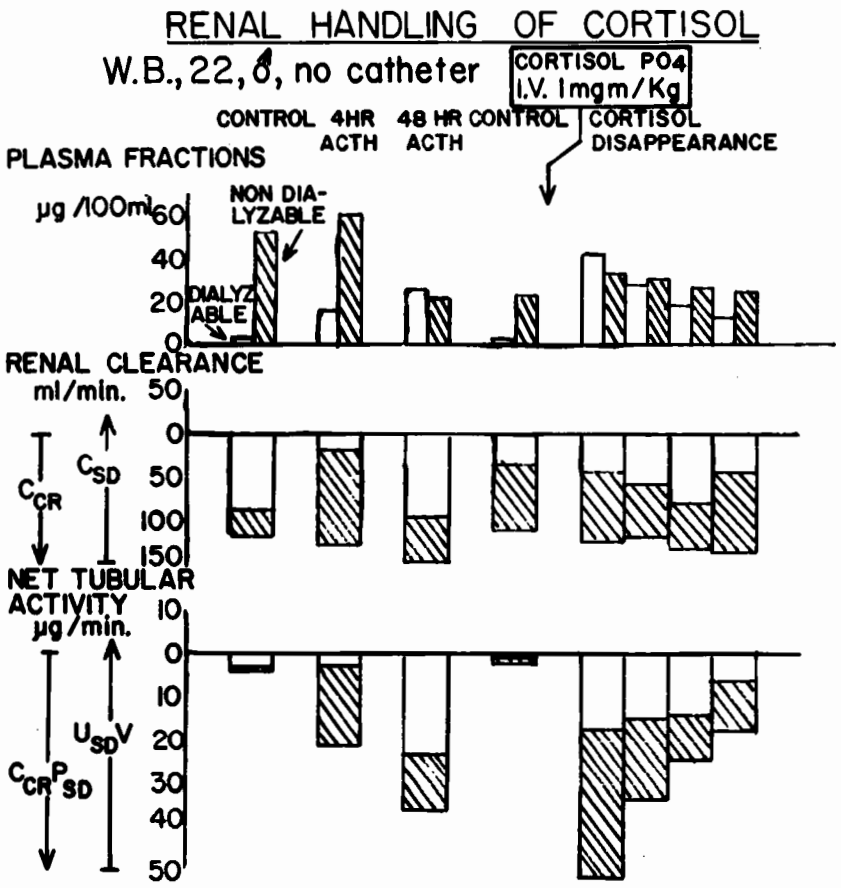
Subject: W.B., 22, male, normal.

1. Plus indicates tubular reabsorption

2. Minus indicates tubular secretion

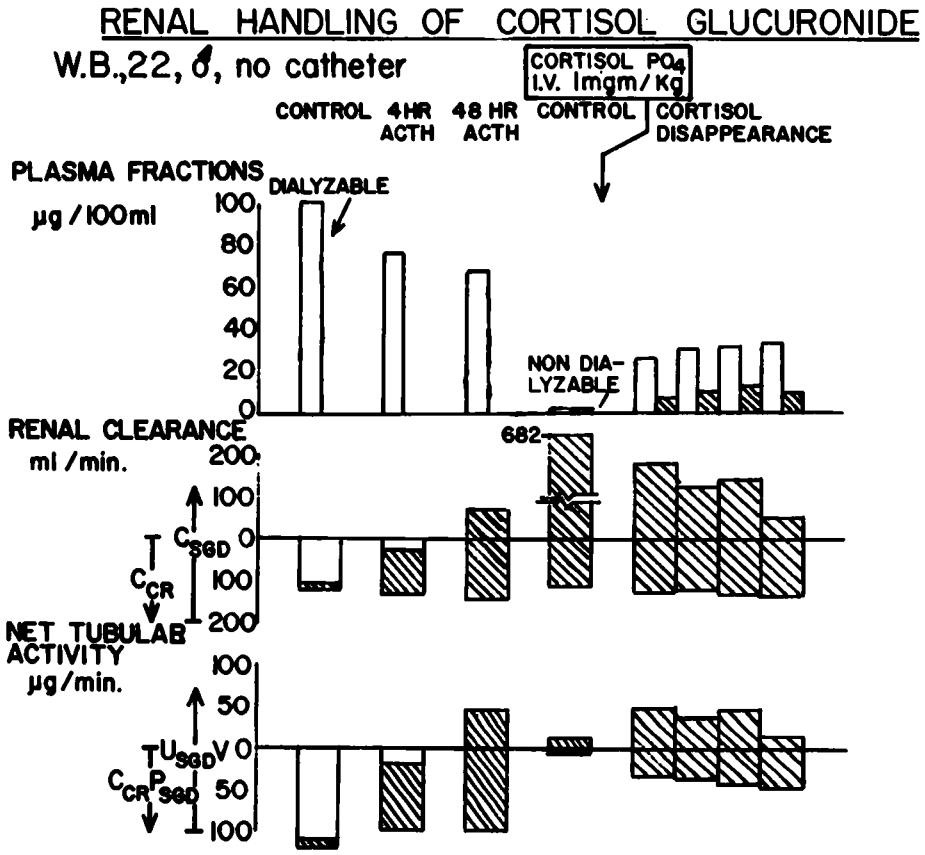
*For explanation see Table XXX.

FIGURE IX "a"



For explanation see Figure VII a.

FIGURE IX "b"



For explanation see Figure VII b.

steroids of the plasma but not of the glucuronide fraction. During the cortisol-phosphate disappearance studies, clearances of the free fraction increased in both subjects while those of the glucuronides increased in only one of them (B.G.). Changes in the renal clearances of either fraction could not be directly related to changes in the plasma levels except to note that the renal clearances of the free fraction increased in each subject as the free-17-hydroxycorticosteroids of the plasma were returning towards normal levels after reaching very high levels due to the administration of a large dose of cortisol-phosphate. Renal tubular secretion of only the glucuronide fraction was demonstrable in 1 of the 2 cases during these studies. The effect of the binding by plasma proteins on pharmacological doses of cortisol would appear to have considerable influence on the plasma disappearance rates and the data derived thereof.

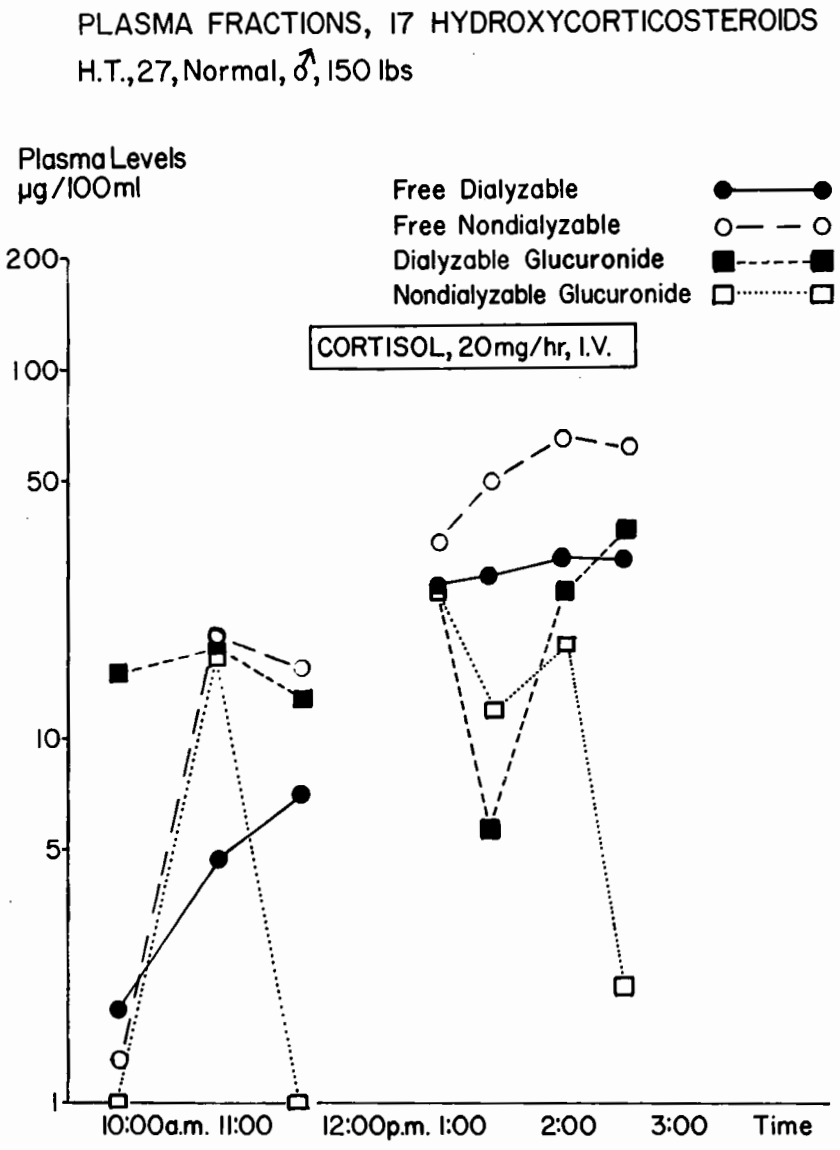
10) Renal Clearance Studies During Constant Infusions of Cortisol, ACTH and Cortisol plus ACTH.

a) Protocol: The previously described clearance techniques were employed. Clearance periods ranged from 16 to 60 minutes in duration; most of the periods were, however, about 30 minutes. The clearance of

exogenous inulin was used to estimate the glomerular filtration rate. For comparison, the clearance of para-aminohippurate was also studied; this was administered through the same infusion. Catheters were used in all 6 subjects and the bladder and catheter were washed out at the end of each clearance period. Free hydrocortisone was employed in all studies. Dr. Robert Morgen collaborated in these clearance studies with the aid of Miss Judith Young, R.N.

Individual variations in the protocol were as follows: (1) In H.T.'s studies there were 4 control clearance periods following which cortisol, 20 mg./hour, was infused at a constant rate by using a constant infusion pump which was employed in this and all subsequent cases. Starting 1 hour after this cortisol infusion there were 4 clearance periods. Uric acid clearances were studied simultaneously during all clearance periods. (2) R.J. was studied during 4 control periods; thereafter, he received a priming dose of cortisol, 80 mg. intravenously, and was started on an infusion of cortisol at the rate of 10 mg./hour. Beginning one hour after the onset of the cortisol administration, there was a sequence

FIGURE X



For explanation see Figure VIII.

TABLE XXXIV a

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Duration min.	V ml./min.	U Free-17-OHCS µg./ml.	U 17-OHCS Glucuronides µg./ml.	U x V Free-17-OHCS µg./min.	U x V 17-OHCS Glucuronides µg./min.
			(Control)			
U_1	32	9.75	.167	1.030	1.63	10.04
U_2	39	12.18	.121	1.057	1.47	12.77
U_3	27	11.66	.072	.983	0.84	11.46
U_4	16	2.50	1.180	4.440	2.95	11.10
			(Cortisol Infusion)			
U_7	32	4.25	5.290	12.260	22.45	52.11
U_8	28	8.57	4.310	-----[1]	36.90	-----
U_9	32	9.69	4.350	6.100	42.20	59.10
U_{10}	29	5.21	5.370	19.780	28.10	103.30

Subject: H.T., 27, normal male.

1. Specimen lost through technical error.

V indicates the minute volume expressed in ml.; U is used to denote the concentration of hormone per ml. of urine and U x V, therefore, stands for the amount of hormone appearing in the urine per minute of the clearance period.

TABLE XXXIV b

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	17-OHCS Levels of the Plasma[1] Nonprotein-bound Free-17-OHCS µg./ml.	Protein-bound Free-17-OHCS µg./ml.		Nonprotein-bound 17-OHCS-Glucuronides µg./ml.	Protein-bound 17-OHCS-Glucuronides µg./ml.
			(Control)		
U_1	(.020)[2]	.017		.155	0.0
U_2	.037	.093		.170	0.0
U_3	.056	.170		.155	0.0
U_4	.068	.154		.135	0.0
			(Cortisol Infusion)		
U_7	.265	.335		.270	.250
U_8	.278	.500		.060	.120
U_9	.300	.610		.180	.160
U_{10}	.310	.620		.320	.047

Subject: H.T., 27, normal male.

1. Values interpolated from semi-log line graph. (Figure X)

2. When no color developed in the dialysate, 0.02 µg./ml. (the lower limit of the method) was used for the calculation of the renal clearance of the nonprotein-bound (dialysable) moieties.

TABLE XXXIV c

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Renal Clearances					
	Nonprotein-bound Free-17-OHCS mls./min.	Total Free-17-OHCS mls./min.	Nonprotein-bound 17-OHCS-Glucuronides mls./min.	Total 17-OHCS-Glucuronides mls./min.	mls./min.	mls./min.
	C_{S-D}	C_{S-T}	C_{SG-D}	C_{SG-T}	C_{IN} [1]	C_{PAH} [2]
			(Control)			
U_1	81	43	65	65	118	558
U_2	40	11	75	75	112	571
U_3	15	4	74	74	104	480
U_4	43	13	82	82	114	497
			(Cortisol Infusion)			
U_7	85	37	193	102	134	578
U_8	132	48	---	---	138	724
U_9	141	46	328	169	136	824
U_{10}	91	30	323	258	132	764

Subject: H.T., 27, normal male.

1. C_{IN} signifies the renal clearance of inulin.

2. C_{PAH} signifies the renal clearance of para-aminohippurate.

TABLE XXXIV d

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Hormone Clearance/Inulin Clearance Ratios[1]			
	C_{S-D}/C_{IN}	C_{S-T}/C_{IN}	C_{SG-D}/C_{IN}	C_{SG-T}/C_{IN}
		(Control)		
U_1	.69	.36	.55	.55
U_2	.36	.09	.67	.67
U_3	.14	.04	.71	.71
U_4	.38	.12	.72	.72
		(Control Infusion)		
U_7	.64	.28	1.4	.76
U_8	.96	.35	---	---
U_9	1.02	.34	2.4	1.2
U_{10}	.70	.23	2.4	2.0

Subject: H.T., 27, normal male.

1. For explanation see Table XXIII.

TABLE XXXIV e

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Filtered Loads		Net Tubular Transport[1]	
	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.
		(Control)		
U_1	2.36	18.29	0.73	8.25
U_2	4.14	19.04	2.67	6.27
U_3	5.82	16.12	4.98	4.66
U_4	7.75	15.39	4.80	4.29
		(Control Infusion)		
U_7	35.50	36.20	13.1	-15.9
U_8	38.40	8.28	1.46	---
U_9	40.80	24.50	-1.4	-34.6
U_{10}	40.90	42.00	12.8	-61.1

Subject: H.T., 27, normal male.

1. Negative value indicates tubular secretion.

TABLE XXXIV f

Renal Handling of the 17-Hydroxycorticosteroids

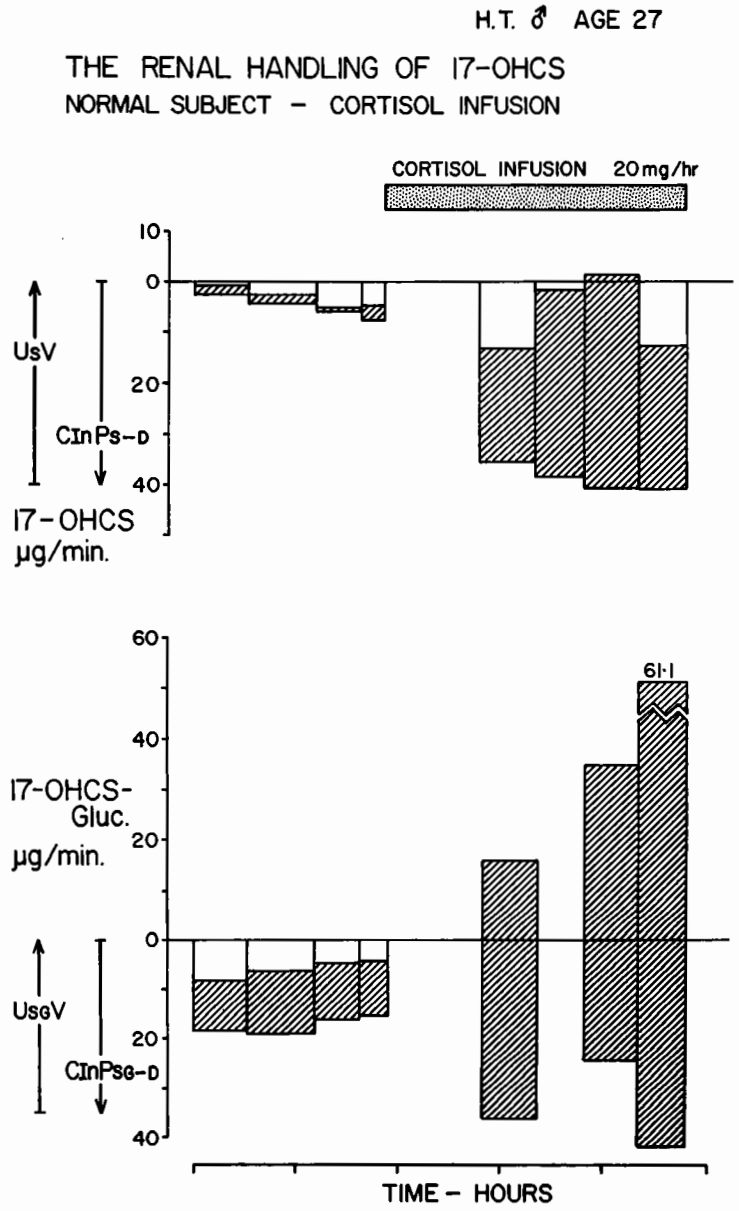
Simultaneous Uric Acid Clearances

Clearance Period	$U_{Uric\ Acid}$ mg./ml.	V mls./min.	$P^{1}_{Uric\ Acid}$ mg./ml.	$C_{Uric\ Acid}$ mls./min.
		(Control)		
U_1	.0691	9.75	.0766	8.8
U_2	.0514	12.18	.0766	8.2
U_3	.0361	11.67	.0766	5.5
U_4	.1246	2.50	.0766	4.1
		(Cortisol Infusion)		
U_7	.0629	4.25	.0752	3.6
U_8	.1043	8.57	.0672	13.3
U_9	.0422	9.70	.0705	5.8
U_{10}	.1238	5.22	.0708	9.1

Subject: H.T., 27, normal male

1. Plasma values interpolated from a semi-log line graph.

FIGURE XI



(No blood levels are shown.)

For explanation see Figures VII a and VII b. Inulin clearances are drawn from the baseline down and hormone clearances from the level of the inulin clearance upward.

of 4 clearance periods. (3) B.M. was followed during 3 control periods. Subsequently she was immediately given ACTH, 20 I.U. intravenously as a priming dose, and an infusion of ACTH at the rate of 5 I.U./hour. Starting 1 hour after the onset of the ACTH, 4 clearance periods were run during the infusion. Uric acid clearance studies were conducted simultaneously throughout. (4) N.R. was studied during 6 periods while cortisol was infused at the rate of 1.2 mg./hour. Subsequently, a second infusion was administered with cortisol given at the rate of 1.2 mg./hour and ACTH at the rate of 6 I.U./hour. Starting immediately after the onset of the second infusion, she was followed through 4 clearance periods. (5) R.D. was followed through 7 clearance periods during which he received cortisol at the rate of 0.6 mg./hour. Then he was given 3 I.U. of ACTH intravenously; this was followed by a second infusion of cortisol, 0.6 mg./hour, and ACTH 6 I.U./hour. There were 5 clearance periods during the second infusion. (6) G.A. received a priming dose of cortisol, 10 mg. intravenously before any study. Then she was followed through 3 clearance periods during which she received cortisol in an infusion at the rate of 3 mg./hour. Next, she was given a priming dose of

ACTH, 20 I.U. intravenously; thereafter, an infusion of cortisol, 3 mg./hour, and ACTH 10 I.U./hour was begun. There was a sequence of 4 clearance periods starting one hour after the beginning of the latter infusion. Uric acid clearance was followed throughout the study.

b) Experimental Subjects: H.T. and R.J. were healthy male medical students, ages 27 and 22 years respectively. B.M. was a 23-year-old healthy female medical student.

N.R. was a 22-year-old white female in good health. About 2 years previously she had undergone bilateral adrenalectomy for Cushing's syndrome. At operation bilateral hyperplasia of the adrenal cortex had been demonstrated.

R.D. was a 30-year-old adrenalectomized-hypophysectomized male in good health. Adrenalectomy had been performed 1 year prior to these studies because of fulminating Cushing's syndrome. A few weeks later an adenocarcinoma of the pituitary was removed at hypophysectomy.

G.A. was a 31-year-old bilaterally adrenalectomized female in good health. Her operations to relieve

Cushing's syndrome had been performed 3 years previously. She had had bilateral hyperplasia of the adrenal cortex.

c) Chemical Methods: The methods for the determination of inulin, PAH and uric acid in both plasma and urine were referred to under part "B" of this section. The 17-hydroxycorticosteroids, both free and glucuronides, were determined as before.

d) Calculation: Renal clearances and related data were calculated as before. The plasma levels of the dialysable free-17-hydroxycorticosteroids and the dialysable 17-hydroxycorticosteroid-glucuronides for the clearance period's midpoint minus 6 minutes was interpolated from a semi-log line graph. Such graphs are shown only in the cases of H.T. and G. A. Figures X, XVI. In order to conserve space the others have been omitted.

e) Results of the Chemical Methods and the Calculations:

(The author has tried to present the results of these renal clearance studies as clearly as possible; however, at times, because of the mass of data distributed through many charts, the reader might easily feel confused. For convenience, if not for clarification, the reader is advised that a summary of the results appears at the end

of this subsection.)

These results are listed in tabular form in Tables XXXIV "a" through XXXIX "f." The values for the renal clearances of inulin, PAH and the nonprotein-bound, as well as the total, hormone fractions are listed in Tables XL "a," XL "b" and XL "c." The mean hormone levels of the nonprotein-bound and protein-bound moieties of the plasma are found in Tables XLI "a" and XLI "b," respectively. In Table XLII are listed the mean percentages of the free fraction which were found to be protein-bound during these studies. Throughout these studies, if significant chromagenic material could not be demonstrated in the dialysate, a value of 0.02 µg./ml. of plasma, the lower limit of the assay, was used in the calculation of the renal clearances of the nonprotein-bound moieties.

Results of the Studies Conducted on H.T. (Tables XXXIV "a" through XXXIV "f"):

The renal clearances of the nonprotein-bound moieties and the total fractions of both the free-17-hydroxycorticosteroids and the glucuronides increased markedly during the cortisol infusion; the mean renal

TABLE XXXV a

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Duration min.	V ml./min.	U Free-17-OHCS µg./ml.	U 17-OHCS Glucuronides µg./ml.	U x V Free-17-OHCS µg./min.	U x V 17-OHCS Glucuronides µg./min.
			(Control)			
U_1	32	5.84	.344	1.645	2.01	9.61
U_2	30	10.83	.189	.616	2.05	6.67
U_3	28	13.21	.171	.775	2.26	10.24
U_4	37	10.32	.190	.779	1.96	8.04
			(Control Infusion)			
U_7	28	11.61	3.850	7.940	44.70	92.20
U_8	30	10.00	4.040	11.230	40.40	112.30
U_9	32	9.68	3.580	17.130	34.70	165.80
U_{10}	30	5.87	4.670	16.250	27.40	95.40

Subject: R.J., 22, normal male.

For explanation see Table XXXIV a.

TABLE XXXV b

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	17-OHCS Levels of the Plasma[1]			
	Nonprotein-bound Free-17-OHCS µg./ml.	Protein-bound Free-17-OHCS µg./ml.	Nonprotein-bound 17-OHCS-Glucuronides µg./ml.	Protein-bound 17-OHCS-Glucuronides µg./ml.
		(Control)		
U_1	.022	.036	.210	0.0
U_2	.035	.035	.022	0.0
U_3	.027	.046	----[2]	0.0
U_4	.014	.072	----[2]	0.0
		(Cortisol Infusion)		
U_7	.630	.580	----[2]	----[2]
U_8	.400	.460	.550	----[2]
U_9	.410	.400	.320	----[2]
U_{10}	.350	.430	----[2]	----[2]

Subject: R.J., 22, normal male.

1. Values interpolated from a semi-log line graph.

2. Specimens lost.

TABLE XXXV c

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Renal Clearances C_{S-D} Nonprotein-bound Free-17-OHCS mls./min.	C_{S-T} Total Free-17-OHCS mls./min.	C_{SG-D} Nonprotein-bound 17-OHCS-Glucuronides mls./min.	C_{SG-T} Total 17-OHCS-Glucuronides mls./min.	C_{IN}[1] mls./min.	C_{PAH}[2] mls./min.
			(Control)			
U_1	91	35	46	46	160	700
U_2	60	29	303	303	150	775
U_3	84	31			155	715
U_4	140	23			147	670
			(Cortisol Infusion)			
U_7	71	37			170	690
U_8	101	47	204		174	735
U_9	85	43	518		172	730
U_{10}	80	35			158	610

Subject: R.J., 22, normal male.

1. C_{IN} signifies the renal clearance of inulin.

2. C_{PAH} signifies the renal clearance of para-aminohippurate.

TABLE XXXV d

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Hormone Clearance/Inulin Clearance Ratios[1]			
	C_{S-D}/C_{IN}	C_{S-T}/C_{IN}	C_{SG-D}/C_{IN}	C_{SG-T}/C_{IN}
		(Control)		
U_1	.57	.22	.30	.30
U_2	.40	.19	2.02	2.02
U_3	.54	.20		
U_4	1.04	.16		
		(Cortisol Infusion)		
U_7	.42	.22		
U_8	.58	.27	1.17	
U_9	.50	.25	3.0	
U_{10}	.51	.22		

Subject: R.J., 22, normal male.

1. For explanation see Table XXIII.

TABLE XXXV e

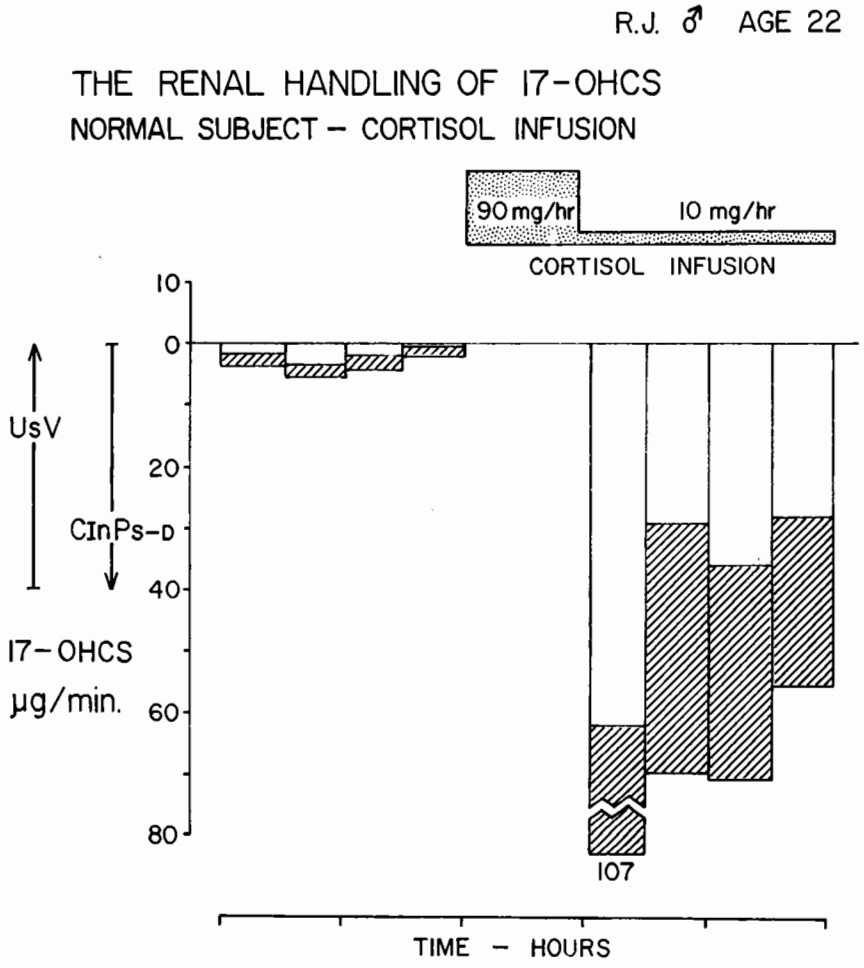
Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Filtered Loads		Net Tubular Transport[1]	
	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.
		(Control)		
U_1	3.52	33.6	1.51	24.0
U_2	5.25	3.3	3.20	-3.4
U_3	4.19		1.93	
U_4	1.91		-0.05	
		(Cortisol Infusion)		
U_7	107		62	
U_8	69.6	95.7	29	-16
U_9	70.5	55.0	36	-111
U_{10}	55.3		28	

Subject: R.J., 22, normal male.

1. Negative value indicates tubular secretion.

FIGURE XII



For explanation see Figures VII a, VII b and XI.

clearance value of the free nonprotein-bound moieties increased from 45 to 112 ml./min., that of the total free fraction from 18 to 40 ml./min., that of the nonprotein-bound glucuronides from 74 to 281 ml./min. and the total glucuronides from 74 to 143 ml./min. The mean clearance for inulin, simultaneously, showed a slight rise from 112 to 135 ml./min. while the mean clearance for PAH changed markedly from 527 to 723 ml./min. The increases of the renal clearances of these hormones' fractions were associated with increased amounts of each fraction appearing in the minute volumes of the urine as well as increases in both their nonprotein-bound and protein-bound moieties appearing in the plasma. As to the free fraction, the nonprotein-bound moiety changed from a mean level of 0.045 to 0.288 µg./ml. of plasma and the protein-bound moiety from 0.109 to 0.516 µg./ml., while the mean nonprotein-bound moiety of the glucuronides rose from 0.154 to 0.210 µg./ml. and that of the protein-bound glucuronides from 0.0 to 0.144 µg./ml. This is graphically shown in Figure X. It should be pointed out that the urinary glucuronide sample for U_8 was lost through a technical error, thus preventing the calculation of the

renal clearance of the fraction for that clearance period. Renal tubular secretion of the glucuronide fraction can definitely be said to have been demonstrated during the U_7, U_9 and U_{10} clearance periods; it was only suggestive for the free fraction in U_9 because its clearance had surpassed that of inulin by only 5 ml./min. These clearances are graphically plotted in Figure XI. The mean uric acid clearance values before and during the cortisol infusion were of the same order: 7 to 8 ml./min.

Results of the Studies Conducted on R.J. (Tables XXXV "a" through XXXV "e"):

The inulin clearances showed a slight increase during the cortisol infusion, rising from a mean control level of 153 to 169 ml./min., but those of PAH fell from a mean control level of 715 to 691 ml./min.; these changes are probably not of significance considering the variability from one clearance period to another. The clearances of the nonprotein-bound moiety of the free fraction were of the same order before, as well as during, the cortisol infusion, with means of 94 and 84 ml./min., respectively; those of the total fraction showed a slight

increase with the means changing from 18 to 40 ml./min. Many of the plasma and dialysate glucuronide samples were lost as a result of a laboratory accident (see Table XXXV "b") but even so, the clearances of the nonprotein-bound moiety of that fraction showed an increase following cortisol with the mean value rising from a control of 175 to 361 ml./min. Both plasma moieties of the free-17-hydroxycorticosteroids showed increases during cortisol with the mean non-protein bound value going from 0.025 to 0.448 µg./ml. and the mean protein-bound value from 0.047 to 0.467 µg./ml. None of the plasma glucuronides were demonstrably protein bound but the nonprotein-bound moiety did rise from a control value of 0.068 to 0.435 µg./ml. The hormone contents of the minute volumes of urine for both fractions markedly increased during the administration of cortisol. Renal tubular secretion of the glucuronides was definitely demonstrable during U_2 and U_9 clearance periods and probably during the U_8 period. The clearance data for the free fraction in this subject is shown in Figure XII.

Results of the Studies Conducted on B.M. (Tables XXXVI "a" through XXXVI "f"):

The inulin and PAH clearances were of the same order before and during the administration of ACTH with the mean inulin clearances at 103 and 107 ml./min,, respectively, and those of PAH at 641 and 635 ml./min. Simultaneously, the clearances of the free nonprotein-bound hormone fraction rose from a mean of 39 to 98 ml./min. while the corresponding means for the total fraction's clearance went from 3 to 10 ml./min. Likewise, the mean for the clearances of the nonprotein-bound glucuronides were 92 before and 133 ml./min. after ACTH while the mean of the total glucuronide clearances went from 79 to 125 ml./min. The amount of each hormone fraction appearing in the minute volumes of the urine after the start of the ACTH rose progressively. The mean values for the plasma moieties showed slight increases for the free nonprotein-bound moiety (0.020 to 0.046 µg./ml.), the free protein-bound moiety (0.218 to 0.259 µg./ml.) and the nonprotein-bound glucuronides (0.105 to 0.170 µg./ml.), but not for the protein-bound glucuronides (0.035 to 0.021 µg./ml.). The highest renal clearance values for the free fraction (U_6 and U_7) and the glucuronides (U_8 and U_9) were not associated with the

TABLE XXXVI a

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Duration min.	V ml./min.	U Free-17-OHCS µg./ml.	U 17-OHCS-Glucuronides µg./ml.	U x V Free-17-OHCS µg./min.	U x V 17-OHCS-Glucuronides µg./min.
			(Control)			
U_1	41	3.71	.122	2.553	.453	9.47
U_2	40	10.35	.067	.876	.693	9.07
U_3	42	8.57	.135	1.192	1.16	10.22
			(ACTH Infusion)			
U_6	47	6.13	.482	1.647	2.96	10.10
U_7	43	5.30	.641	3.344	3.40	17.72
U_8	47	5.13	.619	4.236	3.18	21.73
U_9	46	1.80	1.237	20.260	2.23	36.29

Subject: B.M., 23, normal female.

For explanation see Table XXXIV a.

TABLE XXXVI b

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	17-OHCS Levels of the Plasma[1] Nonprotein-bound Free-17-OHCS µg./ml.	Protein-bound Free-17-OHCS µg./ml.	Nonprotein-bound 17-OHCS-Glucuronides µg./ml.	Protein-bound 17-OHCS-Glucuronides µg./ml.
		(Control)		
U_1	$(.02)^2$	.215	.094	.064
U_2	$(.02)^2$	.22	.120	0.0
U_3	$(.02)^2$	.218	.102	0.0
		(ACTH Infusion)		
U_6	$(.02)^2$	.211	.205	0.0
U_7	.022	.267	.160	0.0
U_8	.048	.27	.118	.023
U_9	.092	.287	.195	0.0

Subject: B.M., 23, normal female.

1. Values interpolated from a semi-log line graph.

2. When no color developed in the dialysate, 0.02 µg. per ml. (the lower limit of the method) was used for the calculation of the renal clearance of the nonprotein-bound (dialysable) moieties.

TABLE XXXVI c

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Renal Clearances C_{S-D} Nonprotein-bound Free-17-OHCS mls./min.	C_{S-T} Total Free-17-OHCS mls./min.	C_{SG-D} Nonprotein-bound 17-OHCS-Glucuronides mls./min.	C_{SG-T} Total 17-OHCS-Glucuronides mls./min.	C_{IN}[1] mls./min.	C_{PAH}[2] mls./min.
			(Control)			
U_1	23	2	101	60	93	634
U_2	35	3	76	76	115	675
U_3	58	5	100	100	102	614
			(ACTH Infusion)			
U_6	148	13	49	49	110	666
U_7	154	12	111	111	109	647
U_8	66	10	184	154	105	635
U_9	24	6	186	186	105	592

Subject: B.M., 23, normal female.

1. C_{IN} signifies the renal clearance of inulin.

2. C_{PAH} signifies the renal clearance of para-aminohippurate.

TABLE XXXVI d

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Hormone Clearance/Inulin Clearance Ratios[1]			
	C_{S-D}/C_{IN}	C_{S-T}/C_{IN}	C_{SG-D}/C_{IN}	C_{SG-T}/C_{IN}
		(Control)		
U_1	.24	.02	1.08	.65
U_2	.30	.025	.66	.66
U_3	.57	.05	.98	.98
		(ACTH Infusion)		
U_6	1.34	.12	.45	.45
U_7	1.42	.11	1.02	1.02
U_8	.63	.095	1.75	1.47
U_9	.23	.06	1.77	1.77

Subject: B.M., 27, normal female.

1. For explanation see Table XXIII.

TABLE XXXVI e

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Filtered Loads		Net Tubular Transport[1]	
	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.
		(Control)		
U_1	1.86	8.74	1.41	- .73
U_2	2.30	13.8	1.61	4.73
U_3	2.04	10.4	.88	0.19
		(ACTH Infusion)		
U_6	2.2	22.55	- .755	12.45
U_7	2.4	17.44	- .999	- .283
U_8	5.04	12.39	1.865	-9.34
U_9	9.66	20.48	7.433	-15.813

Subject: B.M., 23, normal female.

1. Negative value indicates tubular secretion.

TABLE XXXVI f

Renal Handling of the 17-Hydroxycorticosteroids

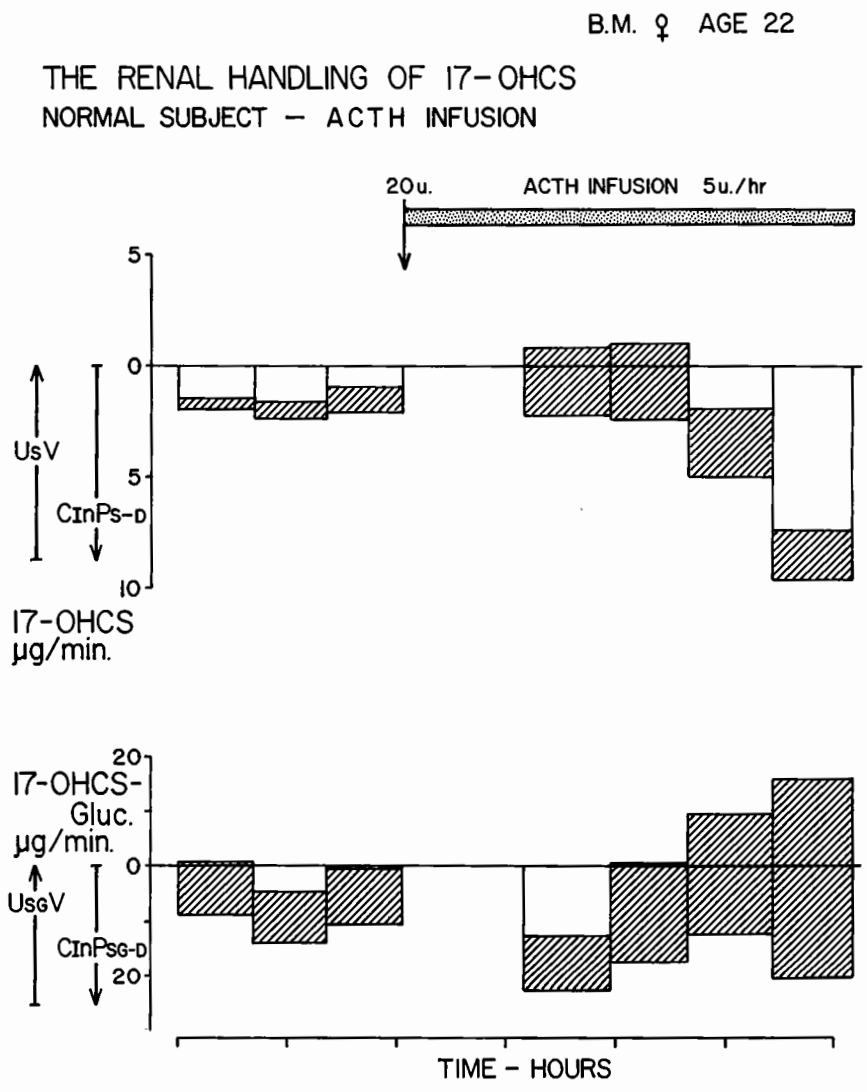
Simultaneous Uric Acid Clearances

Clearance Period	$U_{\text{Uric Acid}}$ mg./ml.	V mls./min.	$P^{1}_{\text{Uric Acid}}$ mg./ml.	$C_{\text{Uric Acid}}$ mls./min.
		(Control)		
U_1	.1317	3.71	.0244	20.0
U_2	.0518	10.35	.0262	20.5
U_3	.0612	8.57	.0266	19.7
		(ACTH Infusion)		
U_6	.0660	6.13	.0296	13.7
U_7	.0610	5.30	.0322	10.0
U_8	.0524	5.13	.0300	9.0
U_9	.1482	1.72	.0296	8.6

Subject: B.M., 23, normal female

1. Values interpolated from a semi-log line graph.

FIGURE XIII



For explanation see Figures VII a, VII b and XI.

highest plasma levels for these hormone fractions. Renal tubular secretion of the free fraction was apparent during the U_6 and U_7 renal clearance periods as it was for the glucuronides during U_8 and U_9. This data has been plotted in Figure XIII. The renal clearances of uric acid during the control periods had a mean value of 23.4 ml./min. and during ACTH a mean value of 10.2 ml./min.

Results of the Studies Conducted on N.R. (Tables XXXVII "a" through XXXVII "e"):

The inulin clearances were of the same degree during the cortisol infusion (mean of 115 ml./min.) and during the infusion of cortisol plus ACTH (mean of 110 ml./min.) while the PAH clearances were correspondingly 469 and 550 ml./min. The renal clearances for the free nonprotein-bound moiety had a mean value of 194 ml./min. during the cortisol infusion and 60 ml./min. during the cortisol plus ACTH infusion, whereas the mean renal clearances for the total fraction remained at the same general level (9 and 14 ml./min., respectively). Simultaneously, the mean clearance value for the nonprotein-bound glucuronides fell from a rate of 231 to one of 123 ml./min. but the mean clearance value for the total glucuronides fell

less (43 to 66 ml./min., respectively). Over the same period of time, the plasma levels of the free hormone fraction changed such that the amount of the nonprotein-bound moiety increased during the second infusion (the mean value changed from 0.020 to 0.051 µg./ml.) and the protein-bound moiety decreased (the mean value fell from a level of 0.412 to one of 0.121 µg./ml.) and, consequently, the total free fraction present in the plasma decreased despite the fact that cortisol was being infused at the same rate during both infusions. Similarly, as to the plasma glucuronide, the nonprotein-bound moiety increased from a mean of 0.053 to one of 0.113 µg./ml. while that of the protein-bound moiety went from 0.304 to 0.113 µg./ml.; the amount of total glucuronides present in the plasma did not therefore change significantly. The amount of the free fraction to appear in the minute urine volumes fell following the start of the second infusion while the amount of the glucuronides to appear continued the progressive increase first observed in U_6, just before the start of the second infusion. Renal tubular secretion could be calculated for the free fraction during the U_1, U_2, U_3 and U_4 clearance periods and

TABLE XXXVII a

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Duration min.	V ml./min.	U Free-17-OHCS µg./ml.	U 17-OHCS-Glucuronides µg./ml.	U x V Free-17-OHCS µg./min.	U x V 17-OHCS-Glucuronides µg./min.
			(Cortisol Infusion)			
U_1	30	5.3	.775	1.222	4.1	6.48
U_2	30	3.83	.982	1.622	3.76	6.21
U_3	30	5.7	.961	1.643	5.48	9.37
U_4	30	5.03	1.236	1.385	6.22	6.97
U_5	60	6.03	.355	3.64	2.14	21.95
U_6	30	1.57	.987	16.56	1.55	26.0
			(Cortisol & ACTH Infusion)			
U_7	30	3.93	.494	4.92	1.94	19.34
U_8	30	1.73	.998	8.52	1.73	17.74
U_9	30	1.47	1.456	4.53	2.14	6.66
U_{10}	30	2.07	1.386	7.28	2.87	15.07

Subject: N.R., 22, adrenalectomized female.

For explanation see Table XXXIV a.

TABLE XXXVII b

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	17-OHCS Levels of the Plasma[1]			
	Nonprotein-bound Free-17-OHCS µg./ml.	Protein-bound Free-17-OHCS µg./ml.	Nonprotein-bound 17-OHCS-Glucuronides µg./ml.	Protein-bound 17-OHCS-Glucuronides µg./ml.
		(Cortisol Infusion)		
U_1	$(.02)^2$	.44	.046	.48
U_2	$(.02)^2$	.43	.046	.385
U_3	$(.02)^2$	.42	.046	.32
U_4	$(.02)^2$	.41	.046	.26
U_5	$(.02)^2$	.39	.048	.19
U_6	$(.02)^2$	.38	.088	.19
		(Cortisol & ACTH Infusion)		
U_7	.019	.15	.135	.19
U_8	.094	.015	.12	.133
U_9	.057	.057	.105	.08
U_{10}	.035	.26	.093	.05

Subject: N.R., 22, adrenalectomized female.

1. Values interpolated from a semi-log line graph.
2. When no color developed in the dialysate, 0.02 µg. per ml. (the lower limit of the method) was used in the calculation of the renal clearance of the nonprotein-bound (dialysable) moieties.

TABLE XXXVII c

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Periods	Renal Clearances C_{S-D} Nonprotein-bound Free-17-OHCS mls./min.	C_{S-T} Total Free-17-OHCS mls./min.	C_{SG-D} Nonprotein-bound 17-OHCS-Glucuronides mls./min.	C_{SG-T} Total 17-OHCS-Glucuronides mls./min.	C_{IN}[1] mls./min.	C_{PAH}[2] mls./min.
			(Cortisol Infusion)			
U_1	205	9	141	12	100	410
U_2	188	9	135	14	110	410
U_3	274	13	204	25	126	427
U_4	311	15	151	23	117	432
U_5	107	5	457	91	122	458
U_6	76	4	295	93	116	457
			(Cortisol & ACTH Infusion)			
U_7	102	11	143	59	115	478
U_8	18	16	123	59	83	369
U_9	38	19	63	37	122	649
U_{10}	82	10	162	108	112	463

Subject: N.R., 22, adrenalectomized female.
1. C_{IN} signifies the renal clearance of inulin.
2. C_{PAH} signifies the renal clearance of para-aminohippurate.

TABLE XXXVII d

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Hormone Clearance/Inulin Clearance Ratios[1]			
	$C_{S\text{-}D}/C_{IN}$	$C_{S\text{-}T}/C_{IN}$	$C_{SG\text{-}D}/C_{IN}$	$C_{SG\text{-}T}/C_{IN}$
	(Cortisol Infusion)			
U_1	2.0	.09	1.41	.12
U_2	1.7	.08	1.2	.13
U_3	2.2	.10	1.6	.2
U_4	2.6	.13	1.3	.2
U_5	.9	.04	3.7	.75
U_6	.7	.03	2.5	.80
	(Cortisol & ACTH Infusion)			
U_7	.9	.10	1.2	.5
U_8	.2	.19	1.5	.7
U_9	.3	.15	.5	.3
U_{10}	.7	.09	1.5	.96

Subject: N.R., 22, adrenalectomized female.

1. For explanation see Table XXIII.

TABLE XXXVII e

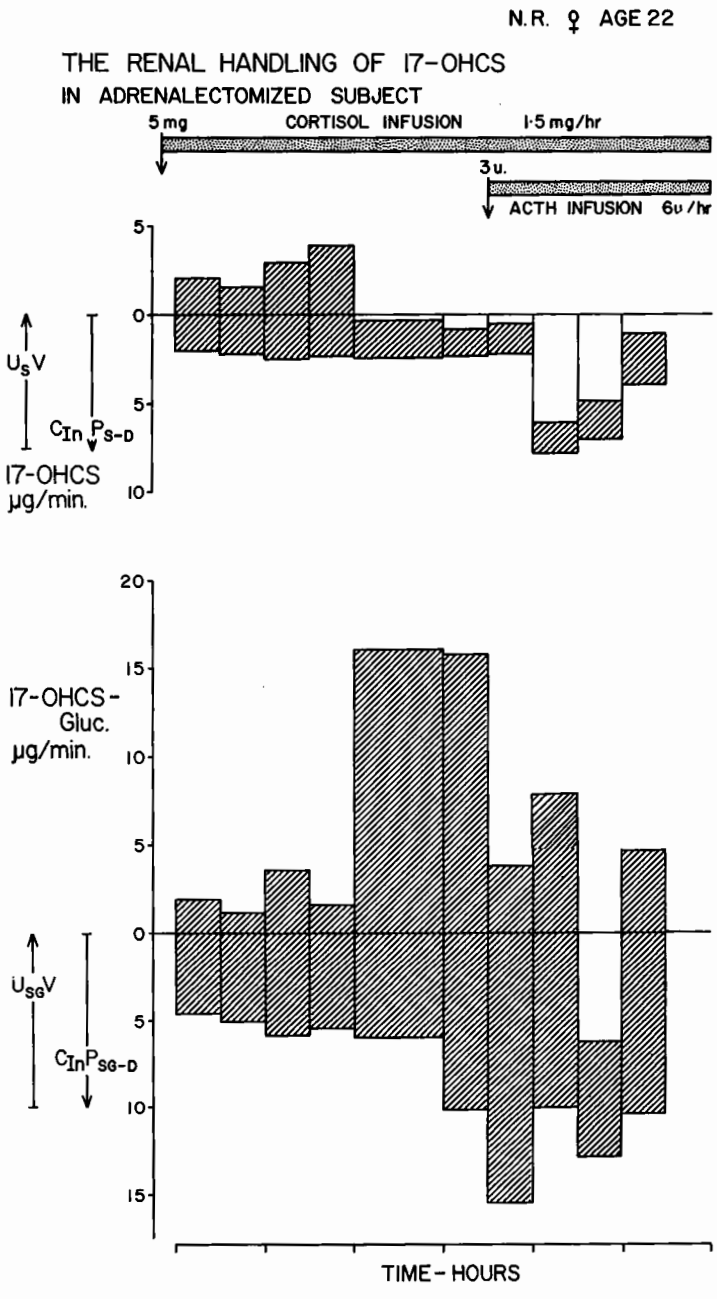
Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Filtered Loads		Net Tubular Transport[1]	
	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.
	(Cortisol Infusion)			
U_1	2.0	4.6	-2.1	- 1.9
U_2	2.2	5.06	-1.56	- 1.2
U_3	2.52	5.8	-2.96	- 3.6
U_4	2.34	5.38	-3.88	- 1.6
U_5	2.44	5.86	0.30	-16.1
U_6	2.32	10.2	0.77	-15.8
	(Cortisol & ACTH Infusion)			
U_7	2.2	15.5	.245	- 3.8
U_8	7.8	10.0	6.1	- 7.7
U_9	7.0	12.8	4.8	6.2
U_{10}	3.9	10.4	1.1	- 4.7

Subject: N.R., 22, adrenalectomized female.

1. Negative value indicates tubular secretion.

FIGURE XIV



For explanation see Figures VII a, VII b and XI.

the glucuronides during those of U_1, U_2, U_3, U_4, U_5, U_6, U_7, U_8. The results have been charted in Figure XIV.

Results of the Studies Conducted on R.D. (Tables XXXVIII "a" through XXXVIII "e"):

The inulin and PAH clearances were of the same order during both the infusion of cortisol alone and cortisol with ACTH; the mean values for the inulin clearances were, respectively, 123 and 126 ml./min. while those for the PAH were 510 and 556 ml./min. The clearances of the nonprotein-bound moiety of the free fraction decreased during the second infusion; they fell from a mean of 23 to 9 ml./min. That of the total free fraction remained low, going from 7 to 2 ml./min. during the first and then the second infusion. Simultaneously, the mean clearance rates for the nonprotein-bound glucuronides decreased slightly, falling from 346 to 296 ml./min., while that of the protein-bound glucuronides remained at the same general level (276 and 296 ml./min., respectively). The concentrations of each fraction of the hormone present in the minute volumes of the urine were of the same order during each infusion. The U_5 urinary glucuronide sample could not be determined in duplicate and, therefore, the

TABLE XXXVIII a

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Duration min.	V ml./min.	U Free-17-OHCS µg./ml.	U 17-OHCS-Glucuronides µg./ml.	U x V Free-17-OHCS µg./min.	U x V 17-OHCS-Glucuronides µg./min.
			(Cortisol Infusion)			
U_1	22	6.86	.0354	1.103	.24	7.57
U_2	21	6.86	.0556	1.284	.38	8.81
U_3	26	7.42	.0742	0.959	.55	7.11
U_4	23	8.13	.0497	1.255	.40	10.2
U_5	15	9.80	.1365	.088	1.34	.86
U_6	16	9.75	.0995	.783	.97	7.6
U_7	31	5.29	.675	1.165	3.58	6.2
			(Cortisol & ACTH Infusion)			
U_8	33	1.45	.0629	.876	.09	12.7
U_9	35	2.11	.182	3.93	.38	8.3
U_{10}	23	5.04	.182	1.102	.92	5.6
U_{11}	28	7.00	.051	.980	.35	6.9
U_{12}	30	5.17	.1022	1.132	.53	5.9

Subject: R.D., 30, adrenalectomized-hypophysectomized male.

For explanation see Table XXXIV a.

Footnote to Table XXXVIII b

Subject: R.D., 30, adrenalectomized-hypophysectomized male.

1. Values interpolated from semi-log line graph.
2. When no color developed in the dialysate, 0.02 µg. per ml. (the lower limit of the method) was used for the calculation of the renal clearance of the nonprotein-bound (dialysable) moieties.

TABLE XXXVIII b

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	17-OHCS Levels of the Plasma[1]			
	Nonprotein-bound Free-17-OHCS µg./ml.	Protein-bound Free-17-OHCS µg./ml.	Nonprotein-bound 17-OHCS-Glucuronides µg./ml.	Protein-bound 17-OHCS-Glucuronides µg./ml.
		(Cortisol Infusion)		
U_1	.17	.029	$(.02)^2$	0.0
U_2	.10	.019	$(.02)^2$	0.0
U_3	.05	.011	$(.02)^2$	0.0
U_4	.05	.024	$(.02)^2$	0.0
U_5	.05	.058	$(.02)^2$	.035
U_6	.05	.105	$(.02)^2$	.06
U_7	.04	.255	$(.02)^2$	.026
		(Cortisol & ACTH Infusion)		
U_8	.03	.17	$(.02)^2$	0.0
U_9	.05	.17	$(.02)^2$	0.0
U_{10}	.064	.23	.025	0.0
U_{11}	.05	.265	.051	0.0
U_{12}	.04	.30	.087	0.0

Footnote to Table XXXVIII c

Subject: R.D., 30, adrenalectomized-hypophysectomized male.

1. C_{IN} Signifies the renal clearance of inulin.

2. C_{PAH} Signifies the renal clearance of para-aminohippurate.

TABLE XXXVIII c

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Renal Clearances C_{S-D} Nonprotein-bound Free-17-OHCS mls./min.	C_{S-T} Total Free-17-OHCS mls./min.	C_{SG-D} Nonprotein-bound 17-OHCS-Glucuronides mls./min.	C_{SG-T} Total 17-OHCS-Glucuronides mls./min.	C_{IN}[1] mls./min.	C_{PAH}[2] mls./min.
			(Cortisol Infusion)			
U_1	1	1	379	379	111	518
U_2	4	3	441	441	107	501
U_3	11	9	356	356	138	544
U_4	8	5	510	510	130	538
U_5	27	12	43	16	126	501
U_6	19	6	380	95	126	456
U_7	90	12	310	135	114	432
			(Cortisol & ACTH Infusion)			
U_8	3	1	635	635	121	526
U_9	7.7	1.7	415	415	129	559
U_{10}	14	3	224	224	131	605
U_{11}	7	1	136	136	136	623
U_{12}	13	2	68	68	123	592

TABLE XXXVIII d

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Hormone Clearance/Inulin Clearance Ratios			
	C_{S-D}/C_{IN}	C_{S-T}/C_{IN}	C_{SG-D}/C_{IN}	C_{SG-T}/C_{IN}
		(Cortisol Infusion)		
U_1	.01	.01	3.41	3.41
U_2	.04	.03	4.12	4.12
U_3	.08	.07	2.58	2.58
U_4	.06	.04	3.92	3.92
U_5	.21	.10	.34	.12
U_6	.15	.05	3.02	.75
U_7	.79	.11	2.72	1.18
		(Cortisol & ACTH Infusion)		
U_8	.03	.004	5.25	5.25
U_9	.06	.01	3.22	3.22
U_{10}	.11	.02	1.71	1.71
U_{11}	.05	.01	1.00	1.00
U_{12}	.10	.01	.55	.55

Subject: R.D., 30, adrenalectomized-hypophysectomized male.

1. For explanation see Table XXIII.

TABLE XXXVIII e

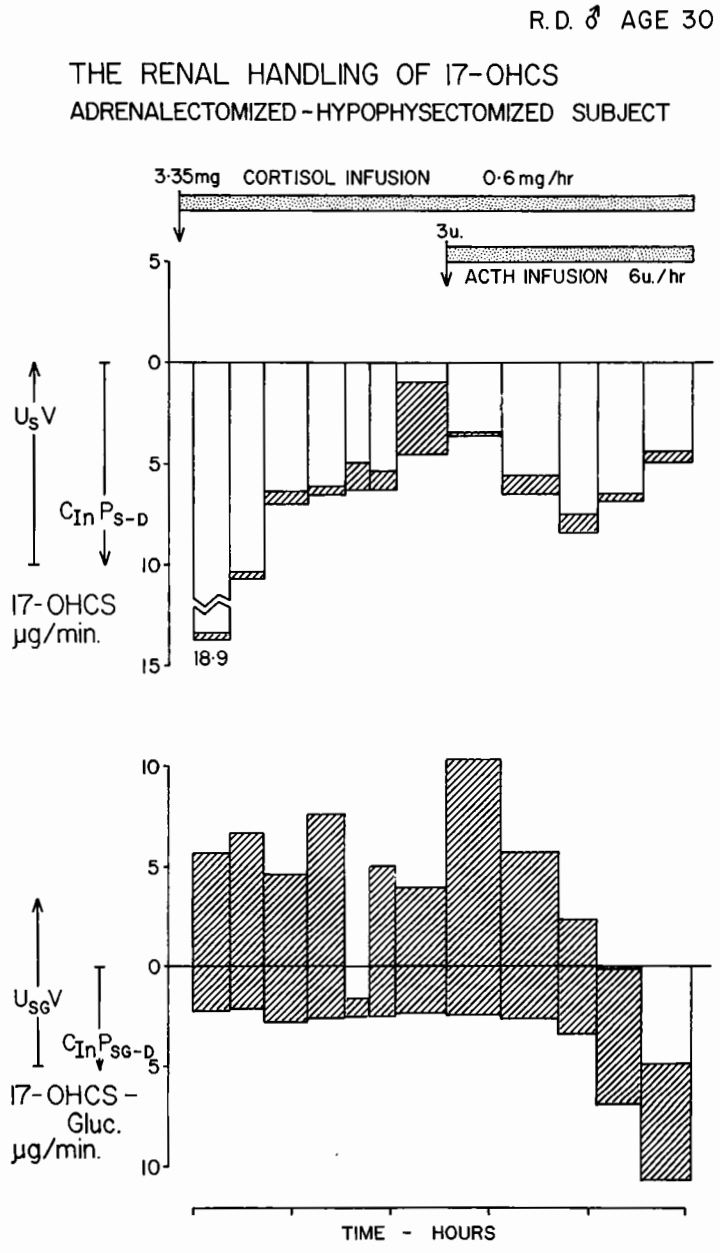
Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Filtered Loads		Net Tubular Transport[1]	
	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.
	(Cortisol Infusion)			
U_1	18.87	2.22	18.6	-5.35
U_2	10.7	2.14	10.3	-6.67
U_3	6.89	2.76	6.3	-4.35
U_4	6.5	2.6	6.1	-7.60
U_5	6.3	2.52	4.96	1.66
U_6	6.3	2.52	5.3	-5.08
U_7	4.56	2.28	.98	-3.92
	(Cortisol & ACTH Infusion)			
U_8	3.63	2.42	3.54	-10.28
U_9	6.07	2.58	5.62	-5.72
U_{10}	8.38	3.28	7.46	-2.33
U_{11}	6.8	6.94	6.45	.036
U_{12}	4.92	10.7	4.39	4.8

Subject: R.D., 30, adrenalectomized-hypophysectomized male.

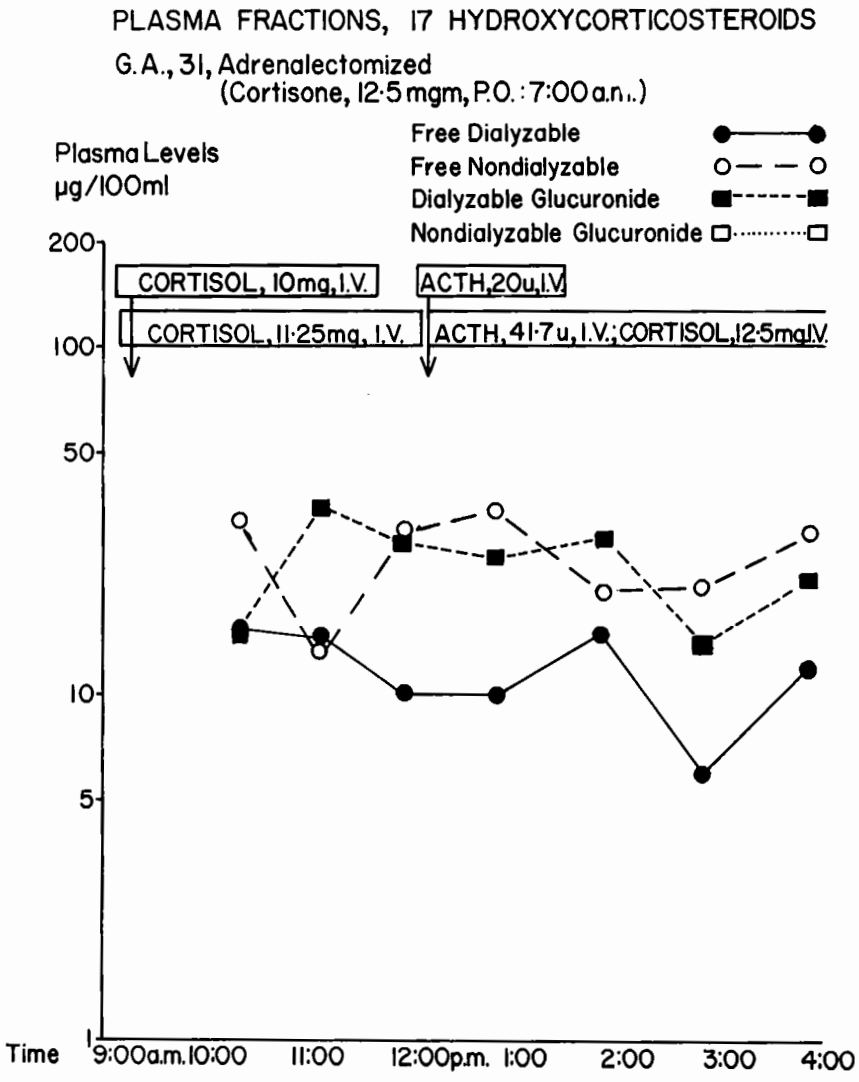
1. Negative value indicates tubular secretion.

FIGURE XV



For explanation see Figures VII a, VII b and XI.

FIGURE XVI



For explanation see Figure VIII.

reliability of such a low urinary glucuronide as 0.86 µg./min. is open to doubt. The plasma levels for the free fraction showed a slight drop in the means of the nonprotein-bound moiety (0.072 to 0.047 µg./ml.) and a moderate increase in those of the protein-bound moiety (0.071 to 0.227 µg./ml.), during the second infusion. Simultaneously, the nonprotein-bound moiety of the glucuronides increased very slightly going from 0.020 to 0.041 µg./ml. and the protein-bound moiety of the glucuronides was never found to be significant. Renal tubular secretion of the glucuronide fraction occurred in clearance periods U_1, U_2, U_3, U_4, U_6, U_7, U_8, U_9, U_{10}. This data is shown in Figure XV.

Results of the Studies Conducted on G. A. (Tables XXXIX "a" through XXXIX "f"):

During both infusions (cortisol and cortisol plus ACTH) the inulin and the PAH clearances were of the same order with the mean clearance of the former remaining at 70 ml./min. and the latter rising only slightly from 286 to 301 ml./min. The mean clearances of the free fraction, both the nonprotein-bound moiety and total fraction, decreased during the second infusion; that of the nonprotein-

bound moiety going from 30 to 12 ml./min. while that of the protein-bound went from 12 to 4 ml./min. The glucuronides were cleared by the kidneys at the same general rate during both infusions; the mean clearances of both the nonprotein-bound moiety and the total fraction went from 98 to 85 ml./min. The amount of each of the hormone fractions appearing in the minute volumes of the urine tended to decrease during the second infusion. The plasma levels of the free fraction showed a very slight decrease in the nonprotein-bound moiety during the second infusion, (0.139 to 0.102 µg./min.), but the protein-bound moiety remained relatively unchanged, going from 0.217 to 0.224 µg./min. The mean of the nonprotein-bound glucuronides of the plasma decreased from 0.258 to 0.206 µg./ml.; no glucuronides were found to be significantly bound to the plasma proteins. Renal tubular secretion of only the glucuronide fraction was demonstrated in clearance periods U_1, U_2, U_3, U_8, and U_9. This data is graphically shown in Figure XVII. The means for the renal clearances of uric acid increased slightly during the second infusion rising from 7.9 to 10.6 ml./min.

The mean degrees of binding of the total frac-

TABLE XXXIX a

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Duration min.	V ml./min.	U Free-17-OHCS µg./ml.	U 17-OHCS-Glucuronides µg./ml.	U x V Free-17-OHCS µg./min.	U x V 17-OHCS-Glucuronides µg./min.
			(Cortisol Infusion)			
U_1	37	4.97	1.22	4.396	6.06	21.85
U_2	37	7.16	.551	3.476	3.95	24.89
U_3	41	3.56	.736	7.091	2.62	25.24
			(Cortisol & ACTH Infusion)			
U_6	39	4.74	.194	4.230	.92	20.05
U_7	42	4.17	.444	1.526	1.85	6.36
U_8	42	3.24	.094	5.337	.30	17.29
U_9	35	3.94	.482	5.745	1.90	22.64

Subject: G.A., 31, adrenalectomized female.

For explanation see Table XXXIV a.

TABLE XXXIX b

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	17-OHCS Levels of the Plasma[1]			
	Nonprotein-bound Free-17-OHCS µg./ml.	Protein-bound Free-17-OHCS µg./ml.	Nonprotein-bound 17-OHCS-Glucuronides µg./ml.	Protein-bound 17-OHCS-Glucuronides µg./ml.
	(Cortisol Infusion)			
U_1	.155	.275	.175	0.0
U_2	.148	.150	.305	0.0
U_3	.114	.225	.295	0.0
	(Cortisol & ACTH Infusion)			
U_6	.137	.225	.273	0.0
U_7	.105	.20	.215	0.0
U_8	.065	.21	.146	0.0
U_9	.100	.26	.191	0.0

Subject: G.A., 31, adrenalectomized female.

1. Values interpolated from semi-log line graph (Figure XVI).

TABLE XXXIX c

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Renal Clearances C_{S-D} Nonprotein-bound Free-17-OHCS mls./min.	C_{S-T} Total Free-17-OHCS mls./min.	C_{SG-D} Nonprotein-bound 17-OHCS-Glucuronides mls./min.	C_{SG-T} Total 17-OHCS-Glucuronides mls./min.	C_{IN}[1] mls./min.	C_{PAH}[2] mls./min.
			(Cortisol Infusion)			
U_1	39	14	125	125	69	290
U_2	27	13	82	82	71	285
U_3	23	8	86	86	69	282
			(Cortisol & ACTH Infusion)			
U_6	7	3	73	73	74	323
U_7	18	6	30	30	72	312
U_8	5	1	118	118	58	257
U_9	19	5	119	119	75	310

Subject: G.A., 31, adrenalectomized female.

1. C_{IN} signifies the renal clearance of inulin.

2. C_{PAH} signifies the renal clearance of para-aminohippurate.

TABLE XXXIX d

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Hormone Clearance/Inulin Clearance Ratios[1]			
	C_{S-D}/C_{IN}	C_{S-T}/C_{IN}	C_{SG-D}/C_{IN}	C_{SGT}/C_{IN}
	(Cortisol Infusion)			
U_1	.57	.20	1.81	1.81
U_2	.38	.19	1.51	1.51
U_3	.33	.11	1.24	1.24
	(Cortisol & ACTH Infusion)			
U_6	.09	.03	.99	.99
U_7	.24	.08	.41	.41
U_8	.08	.02	2.04	2.04
U_9	.25	.07	1.58	1.58

Subject: G.A., 31, adrenalectomized female.

1. For explanation see Table XXIII.

TABLE XXXIX e

Renal Handling of the 17-Hydroxycorticosteroids

Clearance Period	Filtered Loads		Net Tubular Transport[1]	
	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.	Free-17-OHCS µg./min.	17-OHCS-Glucuronides µg./min.
	(Cortisol Infusion)			
U_1	10.70	12.08	4.64	-9.77
U_2	10.51	21.66	6.56	-3.23
U_3	7.87	20.36	5.25	-4.88
	(Cortisol & ACTH Infusion)			
U_6	10.14	20.20	9.22	.15
U_7	7.56	15.48	5.71	9.12
U_8	3.77	8.47	3.47	-8.82
U_9	7.50	14.33	5.60	-8.31

Subject: G.A., 31, adrenalectomized female.

1. Negative value indicates tubular secretion.

TABLE XXXIX f

Renal Handling of the 17-Hydroxycorticosteroids

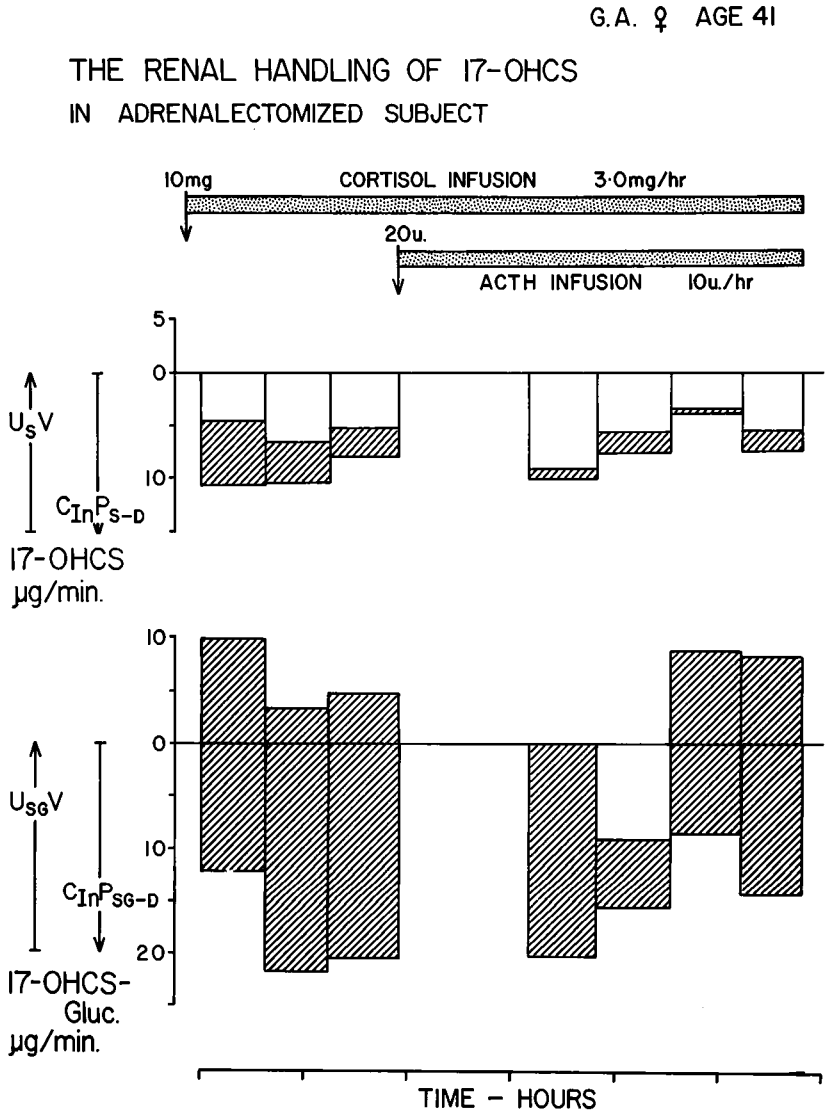
Simultaneous Uric Acid Clearances

Clearance Period	Duration min.	$U_{Uric\ Acid}$ mg./ml.	V mls./min.	$P^{1}_{Uric\ Acid}$ mg./ml.	$C_{Uric\ Acid}$ mls./min.
		(Cortisol Infusion)			
U_1	37	.0956	4.97	.0576	8.2
U_2	37	.0598	7.16	.0581	7.4
U_3	41	.1315	3.56	.0580	8.1
		(Cortisol & ACTH Infusion)			
U_4	35	.4121	1.66	.0573	19.4
U_5	40	.1908	3.55	.0599	11.3
U_6	39	.0816	4.74	.0620	6.2
U_7	42	.1100	4.17	.0623	7.4
U_8	42	.1204	5.65	.0584	11.6
U_9	35	.1043	3.94	.0556	7.4

Subject: G.A., 31, adrenalectomized female.

1. Plasma values interpolated from semi-log line graph.

FIGURE XVII



For explanation see Tables VII a, VII b and XI.

tion of the 17-hydroxycortisosteroids by the plasma proteins are listed in Table XLII. In the three normal controls, the degree of binding fell as the total plasma levels increased to unphysiological values: in H.T. the degree of binding went from 71 percent to 64 percent in the second part of the study; in R.J. from 65 to 51 percent; and in B.M. from 91 to 84 percent. One of the adrenalectomized patients, N.R., showed a decrease in the degree of binding as the total plasma levels fell into physiological ranges (going from 95 to 66 percent) during the second infusion. As to the other two adrenalectomized patients, in R.D.'s case the total free fraction of the plasma rose slightly but the degree of binding increased over 50 percent with the actual change being from 41 to 85 percent; in G.A.'s study the total plasma values remained of the same order and the degree of binding changed but slightly (going from 63 to 70 percent). It should be pointed out that the protein content of these plasma samples was not determined so that any changes in the binding affinity of a given amount of plasma protein were not calculated; this would of course be a more meaningful type of analysis.

A brief summary of the results of the renal clearance studies in these 6 cases would seem to be in order. In none of the subjects were there any marked changes in the means of the inulin clearances during the various parts of the studies. The PAH clearances remained of the same order in 4 of the 6 cases and showed a marked mean increase during the cortisol infusion in one of the normals (H.T.), and a moderate increase in one of the adrenalectomized subjects (N.R.) during the second infusion. The mean renal clearances of the free fraction of the 17-hydroxycorticosteroids increased when the mean plasma levels (total fraction) of the same fraction were high, but it cannot be shown that the highest individual plasma levels were associated with the highest clearance rates. As to the 3 adrenalectomized patients, all of them demonstrated a decrease in the mean renal clearance of the free fraction during the second infusion which contained cortisol and ACTH; in 2 of them the associated mean plasma values (total fraction) increased very slightly but in one of them they decreased. The 3 normals showed an increase in the mean renal clearance of the glucuronides as the plasma levels (total fraction) increased. In the

TABLE XL a

Renal Handling of the 17-Hydroxycorticosteroids

Mean Clearances (mls./min.)

Subject	C_{IN}	C_{IN}	C_{PAH}	C_{PAH}
	Control	Cortisol Infusion	Control	Cortisol Infusion
H.T.	112	135	527	723
R.J.	153	169	715	691
	Control	ACTH Infusion	Control	ACTH Infusion
B.M.	103	107	641	635
	Cortisol Infusion	Cortisol & ACTH Infusion	Cortisol Infusion	Cortisol & ACTH Infusion
N.R.	115	110	469	550
R.D.	123	126	510	556
G.A.	70	70	286	301

TABLE XL b

Renal Handling of the 17-Hydroxycorticosteroids

Mean Clearances of Nonprotein-bound Moieties (ml./min.)

Subject	C_{S-D}	C_{S-D}	C_{SG-D}	C_{SG-D}
	Control	Cortisol Infusion	Control	Cortisol Infusion
H.T.	45	112	74	281
R.J.	94	84	175	361
	Control	ACTH Infusion	Control	ACTH Infusion
B.M.	39	98	92	133
	Cortisol Infusion	Cortisol & ACTH Infusion	Cortisol Infusion	Cortisol & ACTH Infusion
N.R.	194	60	231	123
R.D.	23	9	346	296
G.A.	30	12	98	85

TABLE XL c

Renal Handling of the 17-Hydroxycorticosteroids

Mean Clearances of the Total Hormone Fractions (ml./min.)

Subject	C_{S-T}	C_{S-T}	C_{SG-T}	C_{SG-T}
	Control	Cortisol Infusion	Control	Cortisol Infusion
H.T.	18	40	74	143
R.J.	30	41	175	
	Control	ACTH Infusion	Control	ACTH Infusion
B.M.	3	10	79	125
	Cortisol Infusion	Cortisol & ACTH Infusion	Cortisol Infusion	Cortisol & ACTH Infusion
N.R.	9	14	43	66
R.D.	7	2	276	296
G.A.	12	4	98	85

TABLE XLI a

Renal Handling of the 17-Hydroxycorticosteroids

Mean Nonprotein-bound Moieties of the Plasma

(µg./ml.)

Subject	Free-17-OHCS	Free-17-OHCS	17-OHCS Glucuronides	17-OHCS Glucuronides
	Control	Cortisol Infusion	Control	Cortisol Infusion
H.T.	.045	.288	.154	.210
R.J.	.025	.448	.068	.435
	Control	ACTH Infusion	Control	ACTH Infusion
B.M.	.020	.046	.105	.170
	Cortisol Infusion	Cortisol & ACTH Infusion	Cortisol Infusion	Cortisol & ACTH Infusion
N.R.	.020	.051	.053	.113
R.D.	.072	.047	.020	.041
G.A.	.139	.102	.258	.206

TABLE XLI b

Renal Handling of the 17-Hydroxycorticosteroids

Mean Protein-bound Moieties of the Plasma

(µg./ml.)

Subject	Free-17-OHCS	Free-17-OHCS	17-OHCS-Glucuronides	17-OHCS-Glucuronides
	Control	Cortisol Infusion	Control	Cortisol Infusion
H.T.	.109	.516	0.0	.144
R.J.	.047	.047	0.0	
	Control	ACTH Infusion	Control	ACTH Infusion
B.M.	.218	.259	.035	.021
	Cortisol Infusion	Cortisol & ACTH Infusion	Cortisol Infusion	Cortisol & ACTH Infusion
N.R.	.412	.121	.304	.113
R.D.	.071	.227	.017	0.0
G.A.	.217	.224	0.0	0.0

TABLE XLII

Renal Handling of the 17-Hydroxycorticosteroids

Mean Percentages for the Degree of Protein Binding

of the Free-17-OHCS

Subject	I	II
	Control	Cortisol Infusion
H.T.	71	64
R.J.	65	51
	Control	ACTH Infusion
B.M.	91	84
	Cortisol Infusion	Cortisol & ACTH Infusion
N.R.	95	66
R.D.	41	85
G.A.	63	70

3 adrenalectomized patients, the mean plasma values remained roughly of the same order; yet, in 2 of these subjects there was a definite decrease in the mean clearance rate of the glucuronides during the second infusion. Renal tubular secretion of the glucuronide fraction has been demonstrated to be variably present in all six cases while that of the free fraction has been demonstrated definitely in two (B.M. and N.R.) cases during a total of 6 clearance periods and possibly in another case (H.T.) during one period. Tubular secretion of the glucuronide fraction was more apt to be demonstrable if the plasma levels were high; yet, in two cases (N.R. and R.D.), when the plasma levels were so low as to be less than the lower limit of the method (and there is no reason to believe that these low levels are due to a technical error because serial specimens agree) tubular secretion was calculable.

11) A Study of the Antagonism in the Conjugation of the 17-hydroxycorticosteroids by ACTH.

a) Protocol: Free hydrocortisone 100 mg. in 250 mls. of 5% dextrose and water, was infused from 9:05 a.m. to 9:22 a.m. A control specimen of blood was drawn at 10:30 a.m. using a heparinized syringe. The specimen

was divided into 4 parts. Two parts were saved as duplicate controls and two parts, each consisting of 40 mls. of whole blood, were added to tubes which contained 0.5 I.U. of ACTH. The control blood and the control blood plus the in vitro ACTH were placed in an incubator immediately and kept there for 30 minutes. At 10:32 a.m. 45 I.U. of ACTH was given intravenously over a 15-second period. A second blood specimen was drawn at 11:02 a.m. Thereafter, all blood samples were centrifuged and the plasma was separated. This was then dialyzed as described previously, 20 mls. of plasma against 80 mls. of phosphate buffer, pH 7.4.

b) Experimental Subject: J.B. was a 35-year-old graduate student in good health. Previously, he had demonstrated a marked rise in the 17-hydroxycorticosteroid-glucuronides following 48 hours of ACTH stimulation, Table XXVI.

c) Chemical Methods: The free-17-hydroxycorticosteroid level and the 17-hydroxycorticosteroid-glucuronide level were determined. These were done in duplicate.

d) Standards of Reference: The expected rate

of change in the concentration of these fractions in the plasma and dialysate was estimated from the rates of change observed in the first 30 minutes of the two previously performed cortisol disappearance tests, Figures VI and VIII.

e) Results: The results are listed in Table XLIII. There are discrepancies between the estimated values and the determined values after the administration of ACTH. The greatest difference is between the determined dialysable glucuronide fraction (0.329 µg./ml.) and the corresponding estimated value (0.700 µg./ml.). The other discrepancies are slight. There is also a suggestion of "in vivo" antagonism of conjugation by ACTH.

12. The Distribution of the 17-Hydroxycorticosteroids in Starch Gel Strips following Zone Electrophoresis of Serum.

a) Protocol: Whole blood was drawn in a plain syringe 3 hours after the subject had been given an intramuscular injection of 20 I.U. of ACTH. A clot formed and the serum was separated by centrifugation. The serum was dialyzed against 4 volumes of phosphate buffer, pH 7.4; this buffer was changed 4 times during the 48 hours of dialysis.

TABLE XLIII

Antagonism of 17-Hydroxycorticosteroid Conjugation by ACTH

Sample	Nonprotein-bound Free-17-OHCS µg./ml.	Protein-bound Free-17-OHCS µg./ml.	Nonprotein-bound 17-OHCS Glucuronides µg./ml.	Protein-bound 17-OHCS Glucuronides µg./ml.
Control	.478	.471	.456	0
Control plus in vitro ACTH	.573	.487	.351	0
30 minutes after in vivo ACTH	.437	.550	.329	0
Estimated Change Without ACTH	.380	.420	.700	0

b) Experimental Subjects: J.B. was a healthy 35-year-old graduate student. He previously had demonstrated a brisk rise in the 17-hydroxycorticosteroid level of the plasma soon after intramuscular ACTH.

c) Chemical Methods: Descending starch gel electrophoresis by the Pert modification was performed in 15 mls. of dialyzed serum over a 24-hour period. The gel strip was sectioned and the 17-hydroxycorticosteroid content of the following areas was determined: 1) the first part of the prealbumins from the gamma globulins to the haptoglobins, 2) the last part of the prealbumins from the end of the haptoglobins to the beginning of the albumin, 3) the albumin fraction. The chromagenic content of an appropriate amount of the unused starch gel was also determined.

d) Results: The free-17-hydroxycorticosteroid content of the various sections was as follows:

1. The first part of the prealbumins contained 1.429 µg./sample.
2. The last part of the prealbumins contained 0 µg./sample.
3. The albumin fraction contained 2.740 µg./ml.

The control strip of starch gel contained chromagenic material equivalent to 0.8 µg./sample.

SECTION III

DISCUSSION

A. ANALYSIS OF ACTH TESTS

The analysis of responsiveness in biological systems must allow for variability from one time to another within that system and variability between any two or more systems at any given time. This is certainly true in the assessment of ACTH tests in humans. The great variability of response in normals as judged by the change in the urinary and plasma 17-hydroxycorticosteroids is readily apparent, Tables I "a," III "a," V "a" and VII "a." Liddle et al have shown that the variability in the response of the urinary 17-hydroxycorticosteroids to maximal doses of ACTH (10 or more I.U., intravenously over 8 hours) is about 25% of the mean value on repeated studies (194). In Table III "a" the urinary values in the repeated tests on A.M. and J.B. would tend to confirm this. However, the plasma levels, Table VII "a", during the same tests show greater variation on repeated observations.

With the advent of relatively pure ACTH, as mentioned by Forsham, the route of administration would tend not to be a significant variable if maximal doses of

ACTH are used and provided the subject does not have a hypometabolic condition or edema, as far as using the intramuscular route is concerned (149). We have shown that the responses to intravenous and intramuscular ACTH, in maximal and equal amounts, in the same subject are comparable to the results obtained on the above mentioned tests using intramuscular ACTH during repeated tests (278). We found the variability to be greater in the plasma response than in the urinary response. Different commercial preparations of ACTH have been shown by Eik-Nes to elicit equivalent responses when assayed in humans by the intramuscular route(201).

Several authors have indicated that patients with Cushing's syndrome associated with bilateral adrenocortical hyperplasia were hyperresponsive when tested by ACTH tests of varying design (191, 192, 193, 194, 195 and 196). This finding has been reported to be in marked contrast to the relative lack of response which is attributed to patients with adenocarcinoma and adenoma of the adrenal cortex. Admittedly there are many differences in protocol and methods but, since each group has used their own normal controls, comparison of results is practical.

The results of the studies on patients with adrenocortical hyperfunction which are listed in Tables II "a," IV "a," VI "a" and VIII "a" would appear to disagree with the hypothesis of hyperresponsiveness to ACTH. These include a total of 18 cases; of these, 11 have had histologic examination of the adrenal glands. There are no cases of adenocarcinoma of the adrenal cortex but there are 10 cases of bilateral adrenocortical hyperplasia, one of which was secondary to an adenocarcinoma of the pituitary, and 1 case of unilateral adrenocortical hyperplasia. The responsiveness as judged by the mean percentage change in the urinary values actually indicates hyporesponsiveness in the females with adrenocortical hyperfunction and a normal degree of responsiveness, but one which is prolonged beyond normal duration, in the males. The important thing to emphasize, however, is that the control levels in all cases are significantly elevated; this consistent elevation in the control urinary corticoids has not been reported by others. As to the mean plasma levels, neither the control value nor the 4 and 48-hour responses in the females are significantly different from normal. In the males, although the control level is not abnormal,

the 4 and 48-hour increases expressed in µg./sample are significantly higher than normal while the percentage increase at 48 hours is greater than normal.

When the data of the above mentioned references are analyzed, some of the reasons for these discrepancies become apparent. Liddle reported 1 case of Cushing's syndrome in which the urinary excretion of 17-hydroxy-corticosteroids rose from a control level of 18 mg./24 hours to 57 mg./24 hours after 10 I.U. of ACTH intravenously over 8 hours (191). This represents a percentage increase of 217. The means of the control group rose from 9 to 33 mg./24 hours which is a percentage increase of 267. Christy et al (193), in analyzing the response of the plasma 17-hydroxycorticosteroids to 25 I.U. of ACTH given intravenously over 4 hours, reported an increased sensitivity in 4 cases of Cushing's syndrome with bilateral adrenocortical hyperplasia as compared with the normal response. A case of adrenocortical adenocarcinoma demonstrated a relative lack of response. The mean values of their normal controls rose from 14 to 45 µg./100 mls., a change of 364%, while the mean values for the 4 patients with Cushing's syndrome rose from 32 to 80 µg./100 mls.,

a response of 192%.

Later this same group, in reporting the responsiveness of 14 patients with adrenocortical hyperplasia to the same test, showed that the mean plasma levels went from 30 µg./100 ml. to 81 µg./100 ml. which represents a response of 170 percent, but the mean values in their 15 control subjects had gone from 15 µg./100 ml. to 46 µg./100 ml., a percentile response of 206 (194).

Lindsay et al reported on 3 cases: a patient with adenocarcinoma of the adrenal cortex, 1 with an adenoma and a third with bilateral adrenocortical hypertrophy (195). Only the third was reported to demonstrate an increased responsiveness to 25 I.U. of ACTH, given intravenously over a 6-hour period. However, percentagewise his increase is 300% and that of the mean values of the controls was of the same order at 296%. Nevertheless, Soffer and Laidlaw have reported some cases where the increment of increase percentagewise actually is greater than that of the controls (196, 192).

In speaking of patients with Cushing's syndrome, such terms as "hyperresponsiveness" or "increased sensitivity to ACTH," are misnomers and physiologically

incorrect in many cases. They should not be used. Certainly a few cases do show an increased responsiveness but then so do some normals. All cases of Cushing's syndrome in our series do, however, maintain a greater than normal basal level of urinary excretion and, by inference, an elevated basal glandular secretion rate, regardless of the cause of the disease. Since it is the increased secretion of hormone which is probably responsible for the metabolic changes in the disease, an assessment of the day to day basal activity of the gland would seem to be physiologically more meaningful than determining the degree of response to ACTH stimulation.

Of the cases of Cushing's syndrome listed in the aforementioned tables, 14 out of the total of 18 are females. This sex incidence has been noted by others (1). It is interesting that of these females only 1 presented with fulminating disease, whereas 3 of the 4 men with Cushing's syndrome were severely ill. The reasons for these variations in severity and incidence between sexes are not readily apparent. Estrogens have been shown to delay the plasma disappearance of the 17-hydroxycorticosteroids (272, 254). Furthermore, some of the cases of

Stein-Leventhal syndrome with amenorrhea, sterility, masculinization and enlarged cystic ovaries have been shown to have elevated urinary 17-hydroxycorticosteroids (1). In the women with Cushing's syndrome who are listed in this study, amenorrhea was usually an early and constant complaint. However, Slaunwhite has shown that the degree of protein binding of cortisol by the plasma proteins increases during the last two trimesters of pregnancy when the estrogen and 17-hydroxycorticosteroid levels are rising without being associated with signs of Cushing's syndrome (258). It can be assumed that only the nonprotein-bound moiety of the free-17-hydroxycorticosteroids is biologically active so that, if the increased plasma levels of the hormone appear concurrently with an increase in the affinity of the plasma proteins to bind this hormone, the net biological effect would be unaltered. It is attractive to implicate estrogens in some etiological way with Cushing's syndrome because of these marked sex differences, but variations in their biological effects on the binding of the 17-hydroxycorticosteroids by plasma proteins in these two states are not clear. The interrelationship of estrogen and the 17-hydroxycorticosteroids may well be more complicated than is currently realized.

B. THE EFFECT OF RENAL DISEASE ON THE 17-HYDROXYCORTICOSTEROID LEVELS OF THE PLASMA

An evaluation of the effect of renal failure upon the 17-hydroxycorticosteroid levels of the plasma would seem to indicate that only acute renal failure with absolute or relative anuria can significantly elevate the free or glucuronide fractions, Table IX "a," IX "b" and IX "c." Acute renal failure represents a stressful situation and the adrenocortical hormone production must certainly be greater than that of the normal controls or the relatively calm patients with chronic renal failure; the variability between the groups is not only that of renal function, therefore, and they are not comparable in the strictest sense. Since the greater part of the urinary 17-hydroxycorticosteroids is excreted as glucuronides, it is reasonable that the glucuronide fraction of the plasma is so much higher in some of these cases. From the data it is impossible to say why the free fraction is also elevated in these cases. It could be due to increased hormone production, decreased rate of metabolism or a combination of the two. That the levels of both fractions return to normal very quickly and even before the restoration of normal renal function, as judged

from the serial NPN values of the blood, is quite clear in the data of those patients who have been followed through diuresis and into recovery, Tables X, XI, XII. Certainly any impairment of chronic renal failure cannot account for an elevation of the 17-hydroxycorticosteroids of the plasma as indicated by this data. Similar observations in patients with acute and chronic failure have not been reported previously.

In the few cases of acute renal failure in which the plasma 17-hydroxycorticosteroids were followed during dialysis in the artificial kidney, the free fraction showed a slight rise and the glucuronide level a considerable drop. Tables XIII, XIV, XV. The reasons for this observation are not readily apparent; indeed, several variables are probably at play. Certainly, the glucuronides are less strongly bound to the plasma proteins as shown by Daughaday (232). The dialysis possibly being stressful might trigger an increased production of the free hormone by the adrenal cortex. Secondary to the dialysis the blood volume may have contracted; this could also cause a false elevation in the plasma concentrations. Any of these factors and others not readily obvious might

account for the post-dialysis findings.

C. EVALUATION OF DAUGHADAY'S MODIFICATION OF THE NELSON-SAMUELS METHOD

The Nelson-Samuels method for the determination of small amounts of the 17-hydroxycorticosteroids in urine, plasma and dialysate has been evaluated and found to be satisfactory (136). It is particularly important that the Florisil be very carefully cleaned and heated as shown by Harwood (146). Eik-Nes and Harwood have both mentioned that chloroform should be freshly distilled; the latter recommended double distillation over potassium carbonate (148, 149). The standard deviation for recoveries on urine and plasma has been shown to be between $\pm$15% and $\pm$18%. The lower limit of the assay has been found to be 0.5 µg./sample. For a 20 ml. aliquot of plasma this would represent 2.5 µg./100 mls. From the spectrographic analysis, it is clear that there is an increased absorption of the phenylhydrazine chromagens at 410 mµ. in the hydrocortisone standard, urine, dialysate and plasma (Figures I and II). Such a peak is found only in the eluate made up of methyl alcohol and chloroform in the concentration of 1 part to 4 parts (Figure III). This indicates that the method effectively isolates the 17-hydroxycorticosteroids.

As to the evaluation of the enzyme hydrolysis, the recoveries of tetrahydrohydrocortisone-monoglucuronide in urine are very good (98% ± 2%). This is probably much better than the accuracy which obtains in the routine determination of the samples because the standard deviation for the agreement between samples of a series of urine specimens done in duplicate was ±12%. Incubation studies with proteolytic enzymes, hydrochloric acid at pH 1.5 and β-glucuronidase following the original hydrolysis of the 17-hydroxycorticosteroid-glucuronides would seem to indicate that, in most cases, the original hydrolysis had been complete; significant amounts of chromagenic material were not released in most samples during repeated hydrolysis, Tables XVI, XVII. Furthermore, any chromagenic material so released would need to be isolated and characterized to ascertain whether it contained adrenal corticoids. The nondialysable glucuronides of the plasma were not accurately determined by this method. The absorption spectrum of the phenylhydrazine chromagens in the dialyzed plasma does not peak at 410 mμ., Figure VIII. Frequently, less chromagenic material was found in the dialyzed plasma following equilibrium dialysis

than theoretically should have been present, as indicated by the amount present in the corresponding dialysate. Time did not permit an investigation into the cause of these discrepancies. Plasma is an excellent culture media for microorganisms; urine and dialysate are less so. Since incubation during enzyme hydrolysis was done at 37°C. over a period of 48 hours, it is conceivable that bacteria could have destroyed or impaired the enzyme, destroyed the glucuronides or broken the loose bond between the protein and the glucuronides.

Dialysis would seem to be the least accurate step in the Daughaday method (228). Only three recoveries were done; they gave 62%, 100% and 122% recovery (S.D. $\pm$ 23%). On the few occasions where enough plasma was available for duplicate dialysis the agreement was of the order of 15 to 20%. The fact that the dialysable free fraction during the two disappearance studies fell along a straight line would also indicate that the error is certainly not greater than 20%. No phenylhydrazine chromagens could be extracted from the cellophane following dialysis. Sulfuric acid chromagens were shown to be present in cellophane before dialysis; these usually

disappeared following repeated boiling of the cellophane in distilled water.

The effect of freezing and long term storage upon the 17-hydroxycorticosteroid content of water standards containing hydrocortisone, of urine, and of plasma was investigated. Tables XVIII, XIX, XX and XXI. Within 2 to 3 months after the freezing, there is a demonstrable reduction in the free chromagenic material recoverable from the water standards and the urine. By the end of 8 months, no material was recoverable from a 5 µg. water standard. No chromagenic material could be extracted from the glass of the tubes used for freezing and storage. Thawing was done slowly at room temperature; flash heating which could destroy steroids was not used. No loss of chromagenic activity was observed in the total 17-hydroxycorticosteroids (i.e. small amount of free and large amount of glucuronide) of the urine or in the free fraction of the plasma. Dorfman reported in 1945, when bio-assays were still being used, that freezing did not impair the biological activity of extracts following hydrolysis (83). This would agree with these observations in that the total fraction did not fall with freezing and

storage. Observations of such a loss of free-17-hydroxycorticosteroids in water or urine following freezing and storage has not previously been reported.

D. RENAL CLEARANCE STUDIES ON THE 17-HYDROXYCORTICOSTEROIDS

Equilibrium dialysis, which was used in these studies, has become an accepted technique for separating protein-bound and nonprotein-bound moieties of various substances present in the plasma or serum. The expansion of the volume into which the dialysable moiety is distributed undoubtedly modifies the equilibrium which existed between the two moieties prior to the dialysis. In these studies, however, the ratio of the volume of the plasma sample to the volume of the dialysate is of the order of the ratio of the total plasma volume to that of the interstitial fluid. It has been assumed that the protein-bound moiety is restricted to the plasma volume while the nonprotein-bound moiety is distributed throughout the plasma and interstitial fluid volumes; the degree to which the nonprotein-bound moiety enters the cells is not known. Any errors incurred through disruption of the existing equilibrium between these moieties of the plasma by slight differences in such volume ratios and

by the use of cortisol-free dialysate fluid should be small and constant throughout these studies. Mills, using a newer technique and one which employs the method of ultrafiltration which has been developed by Toribara, has been able to show that as the temperature is lowered the affinity of the plasma proteins for the free-17-hydroxycorticosteroids is increased (262, 279). Since these studies were conducted at 4°C., this undoubtedly has introduced yet another, but constant, error. From the immediately foregoing evaluation of the chemical methods, it is evident that the overall limitations thereof are of the order of ±20% for a 20 ml. sample containing at least 0.5 µg. of chromagenic material. Considering these limitations then, the unphysiological aspects of the method, which have just been discussed, would not seem to be of a degree to alter the overall accuracy of the method and the significance of observations made thereof.

Smith has decreed that a substance which is to be used to measure the glomerular filtration rate must be "physiologically inert" as far as renal function is concerned (275). Ideally, this means that the substance

should not have an effect upon the body which might alter renal function, renal circulation, or the urinary excretion of other substances and that the substance should be neither produced nor metabolized by the kidney. Naturally any other substance, whose renal clearance is to be compared with that of such a substance as would be used to estimate the glomerular filtration rate, should also be expected to meet these criteria. In the strictest sense, such conditions cannot be met by cortisol and cortisone. Raisz et al have shown that patients who have been maintained on a low sodium diet will have a water diuresis and a slight sodium diuresis upon the administration of cortisol (280). They have also shown in humans that following large intravenous doses of cortisol there is an increase in the glomerular filtration rate. Garron et al have reported similar findings in dogs given either cortisol or cortisone (281). These effects have only been observed with doses of cortisol or cortisone that far exceed the usual daily output of the adrenal cortex. In our studies the creatinine and inulin clearances did, on occasion, show slight increases in the presence of high free-17-hydroxycorticosteroids in the plasma and on

2 out of 6 subjects, when it was measured, the PAH clearance (a measure of the renal plasma flow) showed increases during the study: once when the 17-hydroxy-corticosteroids of the plasma had been pushed up by a cortisol infusion and another time when ACTH was used in an adrenalectomized subject and there was no rise in the 17-hydroxycorticosteroids. Nonetheless, when there was a concomitant rise in renal clearance of either of the hormone fractions, such an increase was usually of greater proportions than that of the estimated GFR (glomerular filtration rate) or RPF (renal plasma flow) and, therefore, could not be wholly ascribed to these changes.

Renal tissue from rats has been shown to be capable of metabolizing adrenocortical hormones in tissue incubation studies, but to do so at a much slower rate than that of liver tissue (282, 283). Robbins et al, during rat perfusion studies, were able to demonstrate that there was a significant amount of cortisol metabolized over a 4-hour period in those setups which did contain liver tissue, while the concentration of cortisol in the perfusate of those preparations which contained kidneys but no liver remained unaltered over the same period of

time (284). As to the possibility of synthesis of steroid hormones by the kidney tissue, such work to the best of the author's knowledge has not been done but such a possibility is, admittedly, highly unlikely. Thus, although Smith's criteria cannot be fulfilled in the strictest sense, they would not appear to have been compromised enough to negate significant observations made by these particular chemical methods.

Another theoretical limitation to the interpretation of this data is that we have actually studied the renal clearance of a whole mixture of chromagens which include cortisol and cortisone together with their conjugated reduced derivatives and slight amounts of nonsteroidal chromagenic material. About 60 to 70% of the free chromagenic material in the plasma is cortisol; the rest is mostly cortisone according to the micropolarographic analysis of plasma extracts by Morris and Williams (165). The concentrations of urinary cortisol and cortisone are approximately equal (238). The relative concentrations of cortisol and cortisone conjugates in the plasma are not definitely known but the urinary tetrahydrocortisone-glucuronide (THE-glucuronide) is present in amounts just

about double those of tetrahydrocortisol-glucuronide (THF-glucuronide) (285). Furthermore, during stimulation with ACTH, these proportions of the urinary THF-glucuronide and THE-glucuronide are changed such that THF-glucuronide shows the greatest increment of increase and usually surpassed the concentration of THE-glucuronide (285). For the purposes of this discussion, it will be assumed that cortisol and cortisone are similarly handled by the kidney as is also assumed for the tetrahydro derivatives of these two steroids.

Furthermore, it should be borne in mind that Smith's formula can only be used to calculate the net effect of the kidneys upon the substance being studied. For example, if there should be more than one locus of activity in the renal tubule with reabsorption going on at one spot and excretion at the other, much as is known to happen with potassium, then nothing more than the net effect of such activity can be calculated (275). If the renal clearance of the substance is shown to be greater than that of the estimated GFR, renal tubular secretion is credited for the difference; otherwise, tubular reabsorption would account for those clearances which were less than the GFR.

The earlier data on the renal clearance of the total 17-hydroxycorticosteroids by Bongiovanni, Daughaday and Kornel, indicate that the clearance of the total free fraction is in the range of 5 to 10% of the GFR, while that of the total glucuronides lies between 50 and 150% (231, 228, 233). In the 6 subjects in whom the renal clearances of the total fractions were calculated, we found a slightly wider range for the free fraction, 1 to 35% of the GFR, and for the glucuronide fraction a more narrow and lower range, 10 to 40%. Considering that there were variations in protocol and plasma hormone levels both within our group and in comparison with the work by others, such variations in range are difficult to interpret and may well not be significant, at least for the clearances of the total free fraction.

Despite the limitations which have been discussed above and in some detail, it is apparent from the data that the nonprotein-bound moieties of both the free fraction and the glucuronide fraction, on occasion, are cleared by the kidneys at rates greater than the GFR, thus implying a variable net tubular reabsorption or secretion of these fractions. As to tubular secretion

of the free fraction, it was definitely calculable in 2 out of 10 subjects (B.M. and N.R.) during a total of 6 renal clearance periods and probably calculable in another 2 subjects (W.M. and H.T.) during one renal clearance period for each. Definite tubular secretion of the glucuronides has been demonstrated in 8 out of the 10 subjects during a total of 38 clearance periods. No consistent relationship could be established between the plasma levels of the dialysable moieties or the total fractions of either fraction and the renal clearance rates of these fractions. In the three normal subjects who received the constant infusions, higher plasma levels were generally associated with higher clearance rates but the highest plasma values were not necessarily associated with the highest clearance rates. Some of the highest clearance values for both fractions were calculated

against assumed values for the nonprotein-bound moieties (B.M., N.R. and R.D.); as has been explained previously, when the chromagenic content of the dialysate was so low as to be indeterminable, the lower limit of the method, 0.02 µg./ml., was used in these calculations. This would tend to indicate that the nonprotein-bound moiety does

not determine the degree of tubular secretion and that tubular secretion probably proceeds independently of tubular reabsorption. Whether or not 2 loci are involved as is the case with the renal tubular activity of potassium could not be deduced from this data or this formula as has been pointed out above. No other organic compound has been shown to enjoy bidirectional activity in the renal tubule. Rather recently and akin to these studies, Scheld, using the Toribara ultrafiltration technique which employs centrifugation through cellophane, has shown the renal clearance of the nonprotein-bound free moiety can approach the GFR; and Tom and Voigt, using ultrafiltration techniques which involve filtration through cellophane under pressure, demonstrated that the renal clearance of the free fraction can surpass the GFR after high doses of corticoids (286, 287).

As to the 2 cortisol disappearance studies, the amount of the cortisol phosphate, which cannot be accounted for in the body fluids might well be reversibly bound to the body tissues as suggested by Sheurer and Bondy (247). More of the administered dose can be accounted for in B.G. than in W.B. It should be remembered that B.G. had Cushing's syndrome with truncal

obesity and high urinary and plasma 17-hydroxycorticosteroids. It is conceivable that B.G.'s tissues were already saturated with endogenous cortisol and, therefore, took up the administered cortisol with less avidity than did the tissues of W.B. Such a rationale could also find support in the more rapid disappearance of both of the free moieties in B.G. together with the more rapid appearance of the glucuronides in the plasma. Furthermore, Levin, Daughaday and Brimer have shown that fatty tissues have a relatively low binding affinity for cortisol in the rat and that tissue which contains large amounts of protein binds with much greater avidity (288). Since B.G. obviously had more fat per total body weight than did W.B., her total tissue binding potential would be reduced from that of W.B. on this count also. Because of the satisfactory rate of appearance of the plasma glucuronides in both cases, it has been assumed that neither subject had any defect in the hepatic metabolism of cortisol which could adversely affect the disappearance rate. Data derived from the disappearance rates of pharmacological doses of cortisol would seem to require that the degree of protein binding by the plasma proteins must be taken

into account. This has not previously been reported. It is interesting to consider the variations in the degree of protein binding observed during these studies. Originally, using the same methods, Daughaday reported that he had found a mean of 94% of the free-17-hydroxycorticosteroids of the plasma to be bound to proteins; this is the only published data employing equilibrium dialysis to study the degree of protein binding (228). Later, using radioisotope methods and reverse dialysis, he reported that 99% of the C^{14} labelled cortisol was bound to the plasma proteins at or below the concentration of 1 µg./10 mls. of sample (256). His dialyses were performed at 4°C. Mills reported that at 37°C., with the Toribara technique, 93 to 94% of the plasma corticoids were bound to protein (262). Scheld et al, using the same method as Mills, showed that a range of 75 to 99% of the plasma corticol was bound to protein (286). Some of their total levels were higher than is normally found. They did not, however, comment as to what temperature was employed during centrifugation in their studies. Employing a modification of Daughaday's method, the author found a mean of 27 percent of the plasma free-17-hydroxycorticosteroids to be nonprotein bound or 73 percent

protein bound. The range of the percent of the total free fraction which was protein bound was large, 15 to 96 percent, and the variations in total plasma levels also quite large. Generally, but not consistently, at the higher plasma levels less of the free fraction was bound to protein. ACTH could not be shown to have any consistent effect on the degree of protein binding. As mentioned previously, the determination of the glucuronide fraction in the plasma following dialysis was not found to be dependable; therefore, any conclusions concerning the degree of binding of this fraction would be highly speculative. A mean of 12% of the glucuronide fraction of the plasma was found to be protein bound. The author cannot explain such discrepancies with the published data except to point out that the error of the chemical methods is large and that more precise techniques will need to be developed before we can separate the protein-bound and nonprotein-bound moieties in small samples of plasma.

In the subjects in whom the uric acid clearances were studied, no constant changes were observed. In 1947, Forsham et al reported that the urinary uric acid/creatinine ratio increased with ACTH administration

(289). These patients were studied for longer periods of time than the patients reported here. Also, it had been hoped that something could be learned by comparing the hormone clearances with a substance which showed a high degree of tubular reabsorption. Before any conclusions can be drawn, more studies will have to performed.

Using these methods we are still a long way from really understanding the mechanisms of renal handling which obtain for the adrenal cortical hormones or any other hormones for which we should choose to employ the same methods. In the future, we should look to the development of techniques which would permit the determination of the renal clearances of the individually isolated steroid hormones and in physiological concentrations. Such isolation would involve chromatographic separation of the various steroid hormones present in the plasma as well as the urine. To perform such separation on the small amount of these substances, which are present under physiological conditions, would require the development of radioactive isotope dilution techniques to be used along with the chromatographic separation. Accurate and more dependable methods for the separation of the protein-

bound and nonprotein-bound moieties of the pure hormones will need to be used. The recent use of centrifugation through cellophane membranes and ultrafiltration through cellophane under increased pressure would appear to be a departure in the desired direction. The greatest limitations which will need to be overcome, however, are those associated with the methods employed by renal physiologists. The classical renal clearance formulae employed in clinical studies cannot be used to delineate the renal clearance mechanism of substances which enjoy bidirectional activity in the renal tubule and at more than one site in the tubule. The development of new and more definitive renal clearance techniques is to be hoped for in the future.

E. A BRIEF COMMENT ON THE PRELIMINARY DATA

1) Antagonism of 17-Hydroxycorticosteroid Conjugation by ACTH.

In 1954, Sandberg studied patients with reference to their adrenocortical responsiveness to ACTH and cortisol disappearance before and during surgical stress (209). During the stress, he found increased adrenocortical responsiveness and prolongation of cortical disappearance. Moore confirmed such an increased responsiveness during

surgical stress in 1957 (214). More recently Eik-Nes has reported a prolongation of cortisol disappearance in stressed dogs (290). Melby has made similar observations in patients in bacteremic shock (291).

During some of the studies which are being presented in this thesis, certain cases, W.M., W.B. and B.G., showed decreased plasma 17-hydroxycorticosteroid-glucuronide levels following 4 and 48 hours of ACTH administration (20 I.U. intramuscularly every 6 hours). Tables XXII, XXIV, XXVIII and XXXI "b." The observation was repeated on W.B., Table XXXI "b." During the studies employing constant infusions, B.M. and G.A. (total adrenalectomy) showed decreased levels of plasma glucuronides following the start of ACTH.

The acute study reported in Part II of Experiments and Results was designed to ascertain whether a large dose of ACTH might impair the rate of increase in the plasma glucuronides during the early part of a cortisol disappearance test and possibly offer an explanation as to why cortisol disappearance is prolonged in stress. Such an impairment in the rate of conjugation was observed. However, because of the limitation of time, the subject

studied was not his own control. For confirmation, the test should be repeated several times and each subject should act as his own control. Berliner has recently described prolongation of cortisol disappearance in one subject receiving a large dose of ACTH (292).

2) The Distribution of the 17-Hydroxycorticosteroids in Zone Electrophoresis of Serum.

Slaunwhite and Sandberg, employing boundary electrophoresis, have defined a specific alpha globulin as having a high binding affinity for C^{14} labelled hydrocortisone (258). If this protein is to be further identified and characterized, other means of separation will have to be found because there is layering of various proteins in boundary electrophoresis (273). Starch gel zone electrophoresis can be used for such separation (273). Currently only small amounts of serum are employed in this method (0.02 mls.). Pert is developing a large sample technique (10 to 20 ml./sample). When the method becomes practical, it could afford an opportunity to isolate and characterize this particular protein by using endogenous as well as exogenous hormone. The preliminary studies herein reported suggest that there is a zonal distribution of the 17-hydroxycorticosteroids in the starch gel and

that with refinements the method could be quite useful.

The migration site of transcortin in zone electrophoresis is not known. Pert reports that the alpha globulins migrate with the prealbumins and the albumin fraction (293). The color in the first part of the prealbumins might be due to hormone which is bound to transcortin. Certainly there is no binding by any protein traveling in the second part of the prealbumins. The color in the albumin fraction could be due to that of the hormone which is bound to albumin or to some specific protein traveling with the albumin or to a combination of both.

SUMMARY

1) The various bio-assays and chemical methods which have been developed to measure corticoid activity in the human and the knowledge gained by their use have been reviewed.

2) A statistical analysis of the response in the urinary and plasma 17-hydroxycorticosteroids to ACTH in normal controls and in patients with adrenocortical hyperfunction has been presented. Patients with Cushing's syndrome in association with adrenocortical hyperplasia do not characteristically demonstrate a greater than normal response. All patients in this study with adrenocortical hyperplasia do show a characteristically elevated basal urinary excretion of the 17-hydroxycorticosteroids.

3) The free-17-hydroxycorticosteroids and the 17-hydroxycorticosteroid-glucuronides are significantly elevated in acute renal failure. Both fractions were found to have normal levels in chronic renal disease.

4) A thorough evaluation of the Daughaday modification of the Nelson-Samuels method for the determination of the free and glucuronide fractions of the 17-hydroxycorticosteroids in plasma, dialysate and urine

is presented. The method was found acceptable except in the determination of the protein-bound moiety of the glucuronides in the plasma. Freezing and storage of water and urine with added hydrocortisone for greater than two months has been shown to lead to a loss of chromagenic activity in the water and urine.

5) Renal clearance studies of the 17-hydroxycorticosteroids on 11 individuals are reported in detail. Variable net renal tubular reabsorption and secretion for both the free and glucuronide fractions were observed. Clearance values and renal tubular activity have been calculated for the free and glucuronide fractions.

6) In two cortisol disappearance studies the nonprotein-bound and protein-bound moieties of the free-17-hydroxycorticosteroids were shown to disappear at different rates; the disappearance half time of the nonprotein-bound moiety was 84 and 96 minutes; that of the protein-bound moiety was 244 and 390 minutes.

7) In one case a large intravenous dose of ACTH has been shown to antagonize the rate of conjugation of cortisol. It is suggested that this mechanism could account for the prolongation of the cortisol disappearance rates in stress.

8) A preliminary report on the use of zone electrophoresis to study the zonal distribution of the protein-bound 17-hydroxycorticosteroids is presented.

ADDENDUM

Following the typing of this thesis, two articles defining a renal tubular mechanism which could possibly be utilized by the 17-OHCS have come to the writer's attention. These are: 1) Weiner, I.M., Washington, J.A., II, Mudge, G.H.; Studies on the renal excretion of salicylate in dogs. Bul. Johns Hopkins Hospital, 105: 284, 1959. and 2) Weiner, I.M., Washington, J.A., II, Mudge, G.H.; On the mechanism of action of probenecid on renal tubular secretion. Bul. Johns Hopkins Hospital, 106: 333, 1960.

These authors have presented evidence that salicylates and probenecid are actively secreted by the proxinal renal tubule and that this is associated with reabsorption from the tubular fluid of the free unionized acidic forms in the distal tubule. This reabsorption would seem to depend upon the gradient for the free acid between the interstitial fluid and the fluid in the renal tubule. Thus, the cell membrane of the renal tubular cells is viewed as being lipoidal and the degree of tubular reabsorption would vary as the degree of lipid solubility which is determined by the urine pH and volume.

Tubular excretion of probenecid was demonstrably blocked by PAH; this can be thought of as competitive suppression of tubular secretion of one compound by another, both compounds being secreted by the same mechanism.

During the administration of probenecid, Tom and Voigt report that renal clearances of the nonprotein-bound free 17-OHCS increase to levels higher than the GFR and Daughaday has shown that the clearance rates for the glucuronides are decreased below the pretreatment control levels. (287, 228) These observations would suggest that the free chromagens are subject to a different renal tubular mechanism than are the glucuronides.

Cortisol and cortisone, the free Porter-Silber chromagens, have pKa values and lipid solubility coefficients which are extremely close. The same would hold for tetrahydrocortisone-monoglucuronide and tetrahydrocortisol-monoglucuronide, the two significant conjugated chromagens. The free chromagens are reabsorbed to a high degree in the renal tubule and they are known to be highly soluble in lipids. The conjugated chromagens are reabsorbed to a lesser degree and net tubular secretion may

even be demonstrated for them; they are known to have a low degree of lipid solubility. It is worth considering the proposition that the differing renal tubular activities of the free and conjugated adrenal corticoids is significantly related to the differences which exist between their lipid solubility coefficients of their constituent chromagens.

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