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ADRENOCORTICAL STEROID PROFILE IN
THE HYPERTENSIVE DOG

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

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The Ohio State University
1961

Approved by

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DEDICATION

This work is dedicated to my wife, Dr. Norma F. Besch. After having completed her graduate training, she was once again subjected to almost social isolation by the number of hours I spent away from home. It is with sincerest appreciation for her continual encouragement that I dedicate this to her.

ACKNOWLEDGMENTS

I wish to acknowledge the assistance and encouragement of my Professor, Doctor Katharine A. Brownell. Equally important to the development of this project are the experience and information obtained through the association with Doctor Frank A. Hartman, who over the years has, along with Doctor Brownell, devoted his life to the development of many of the techniques used in this study.

It is also with extreme sincerity that I wish to acknowledge the assistance of Mr. David J. Watson. He has never complained when asked to work long hours at night or weekends. Our association has been a fruitful one.

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Also in order is a note of thanks to Miss Margaret J. Kegerreis of the Department of English, who read the entire manuscript for grammatical errors. Equally important is a vote of thanks to Mrs. Roger Engle for typing the entire manuscript.

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List of Abbreviations

Abbreviation	Meaning
a or α	alpha; behind molecule
b or β	beta; above molecule
BTZ	blue tetrazolium
C/F	chloroform:formamide
CHCl_3	chloroform
CrO_3	chromic acid
DNEH	dinitrophenylhydrazine
E_2B	Eberlein-Bongiovanni system
ETAC	ethyl acetate
ETOH	ethanol
HAc	acetic acid
(I)	inflection
K or diK	ketal or diketal
max	maximum
MECl_2	methylene dichloride
MEOH	methanol
min	minimum
M.P.	melting point
MnO_2	manganese dioxide
M/PG	methylcyclohexane:propylene glycol
NaBiO_3	sodium bismuthate
ol	hydroxyl group

List of Abbreviations (continued)

Abbreviation	Meaning
one	carbonyl group
p	probability
p-TSA	p-toluenesulphonic acid
TH	tetrahydro
T/PG	toluene:propylene glycol
TPTZ	triphenyl tetrazolium chloride
UV	ultraviolet

CHAPTER I

GENERAL INTRODUCTION AND CONSIDERATIONS

The work reported herein stems from a larger undertaking by Doctors Katharine A. Brownell and Frank A. Hartman of the Department of Physiology, Ohio State University Medical School.

For thirty years this group has investigated adrenal physiology. Several years ago they reported data indica-

K. A. Brownell, F. A. Hartman and T. Y. Liu, Amer. J. Physiol., 167:605, 1951.

F. A. Hartman and T. Y. Liu, Fed. Proc., 9:57, 1950.

ting the existence of a bioassayable hormone which seemed not to be under the control of ACTH, yet this substance was elaborated by the adrenal cortex and was obtained by an organic solvent extraction. This hormone was obtained from beef adrenal glands.

Concurrently in their laboratories, other projects concerning the relationship of the adrenal to hypertension were being carried out. They found that organic solvent extracts of adrenal venous plasma from hypertensive dogs, when bioassayed by their technique, indicated the possible existence of a hormone similar to that found in beef glands. In contrast, normal controls usually failed to produce this

response as measured by the bioassay.

The problem of this report evolved from the above studies carried out by Hartman and Brownell. Many studies had been made to determine if blood from hypertensive patients differed in steroid content from that of normotensive patients. The claims in the literature are conflicting. However, these studies reflect only the total content of di-hydroxy-acetone type steroids and therefore fail to give a true picture of the adrenocortical steroid profile.

Since the organic solvent used for the extraction preferentially partitioned the steroids from the blood plasma, along with a small amount of very polar lipids, the possibility of this hormone being protein in nature was eliminated. The necessity of fractionating and quantitating these extracted steroids was obvious.

In order to obtain sufficient material to conduct this type of adrenal cortical steroid fractionation, large volumes of adrenal venous blood were collected from both normotensive and experimental renal hypertensive dogs. Initial extraction procedures were carried out on the adrenal venous plasma from normotensive and hypertensive animals. These extracts were chromatographed. An area (BF), more polar than cortisol, was observed, as well as the usual cortisol and cortisone-like areas. This BF area was markedly increased in the hypertensive dog blood, while

in the normotensive dog blood it was present only in trace³ amounts. Therefore, it seemed of paramount importance to elucidate the structures of the component steroids.

The nature of this report is therefore primarily limited to:

I. the isolation and identification of the adrenocortical profile of the BF areas of,

A. Normotensive dogs

B. Hypertensive dogs.

II. synthetic preparation of the isolated compounds in quantities sufficient to be used in the identification work and for future studies in the laboratories of Hartman and Brownell.

CHAPTER II

HISTORICAL REVIEW

Hypertension

Hypertension is not a new disease. It was known to ancient physicians, although its various clinical manifestations, as they are recognized today, were usually thought to represent separate and distinct entities.

Physiological concepts of hypertension, which are today the subject of argument, began in 1830, after the development of the first useful sphygmomanometer by Herison. However, it was not until the year 1880 that Von Basch investigated hypertension. These experiments have been described by Major.

R. H. Major, Classic Descriptions of Disease, Springfield, Charles C. Thomas, 1932.

Just prior to this time, extensive physiological investigations by Ludwig, Macey and Traub had laid the ground work which Von Basch had applied to the disease. It was Traub, in the year 1856, and Kirkes, one year later, who were probably the first to show that elevated blood pressure might be the result of increased arterial tension, although they considered an increase in the action of the heart the primary cause.

The etiology of hypertension is yet today unsettled;

there are many factors operating etiologically, with one or the other predominating.

Adrenal Cortical Hormones

Concurrent with the developments in vascular disease, while completely unrelated to hypertension, the studies of the endocrine system and particularly that of the adrenal gland were being carried out. Perhaps the earliest recognition of a hormonal contribution to cardiovascular changes is that encountered in the now classical description, by Addison in the year 1855, of the syndrome which now bears his name. His description of a terminal patient with hypo-adrenal corticalism is essentially that of a patient in circulatory collapse. However, many years passed before the adrenal was given a role in the maintenance of blood pressure. In 1895, Oliver and Shaffer demonstrated that an extract of the adrenal gland, when injected into animals, raised the blood pressure. Thus, the chasm between cardiovascular disease and endocrinology had been bridged. For many years the adrenal was studied regarding its role in the production of catecholamines. This was considered its primary function.

It was not until the late 1920's and early '30's that the adrenal suddenly took on a new-found importance. Although its role of being essential to life in animals and humans was recognized, no single basis for this importance could be demonstrated.

The first extracts of adrenal cortical tissue which significantly prolonged life in the adrenalectomized animal were prepared in 1927 by Hartman and co-workers.

F. A. Hartman, C. G. MacArthur and W. E. Hartman.
Proc. Soc. Exptl. Bio and Med. 25:69, 1927.

Later Swingle and Pfiffner also prepared extracts. This

J. J. Pfiffner and W. W. Swingle, Anat. Rec. 44:225,
1929.

active agent was designated "Cortin" by Hartman, which term subsequently became ambiguous in meaning when investigations led to the isolation of many pure chemical compounds. The isolation and chemical characterization of these cortical hormones, the result of intensive investigation was undertaken in 1934 primarily by four groups of investigators. These four principle investigators were E. C. Kendall, J. J. Pfiffner, and O. Wintersteiner in the United States and T. Reichstein in Switzerland. Several crystalline compounds were quickly obtained by each group, and designated, in the order of their isolation, by the letters of the alphabet. However, physiological activity was not clearly associated with a crystalline product until 1936, when Kendall's group established the effectiveness of their

H. L. Mason, L. S. Myers and E. C. Kendall, J. Biol. Chem., 116:267, 1936.

compound E, known today as Cortisone. Subsequently reports followed from the other groups announcing their isolation of the same compound. Shortly thereafter the structure of this compound was established. Following this identification, mainly carried out in the laboratory of Reichstein, there remained the synthesis of these compounds. The first to be prepared artificially was 11-deoxycorticosterone. This was obtained by Reichstein in 1937 in fair yield from cholesterol or stigmasterol as the original starting material. Since this was the first biologically active corticoid, it immediately began to be investigated by many workers. Since it was observed to prevent circulatory collapse in the adrenalectomized animal, it was immediately linked with cardiovascular changes, and many studies were carried out in this field.

Hypertension and the Adrenal Cortical Hormones

It is quite obvious that it is impossible to discuss the etiological, pathological and physiological factors contributing to hypertension. Equally impossible would be a review of the literature regarding the endocrines in general and their involvement in hypertension. However, it would seem in order, because of the nature of this

work, to examine critically the role of the adrenal corticoids as to their production, secretion and metabolism relate to hypertension. Relatively conclusive evidence characterized the initial biological studies with certain steroids, commonly termed mineralocorticoids, implicating them in the genesis of hypertension as shown by Ferrebee, Loeb, Kuhlmann, Grollman, Selye and Friedman.

J. W. Ferrebee, C. Ragan, D. W. Atchley and R. F. Loeb, J.A.M.A., 113:1725,1939.

R. F. Loeb, D. W. Atchley, D. W. Ferrebee and C. Ragan, Tr. A. Am. Physicians, 54:285, 1939.

D. Kuhlmann, C. Ragan, J. W. Ferrebee, D. W. Atchley and R. F. Loeb, Science, 90:496,1939.

A. Grollman, T. R. Harrison and J. R. Williams, J. Pharmacol. and Exper. Therap., 69:149,1940.

H. Selye, C. E. Hall and E. M. Rawley, Canad. M. A. J., 49:88,1943.

S. M. Friedman, C. L. Friedman and M. Nakashima, Am. J. Physiol., 163:319,1960.

In the light of present day knowledge much of the early work appears poorly conceived, and carried out. Many of these early studies can be summed up by presenting a typical study. Goldman and Schroeder studied the relation of the intravenous use of desoxycorticosterone to the blood

M. L. Goldman and H. A. Schroeder, Amer. J. Med., 5:33
1948.

pressure response in normotensives and hypertensives. They were able to demonstrate that the intravenous use of DOCA led to an immediate pressor response in hypertensive patients which was not produced in the normotensive patients. As a control measure, other steroids in the same solvent failed to produce this response in the hypertensive group. Secondly, they used DOC-glucoside (thus inactivating the alpha, beta-unsaturated carbonyl of ring A) and failed to find a significant difference between the normotensive and hypertensive patients.

In retrospect, we may say, by inference, that Pardee

I. Pardee, Am. J. Sci., 119:1,1935.

was the first to correlate increased adrenal corticoids with hypertension when he studied basophilic hyperplasia of the pituitary in its relation to essential hypertension.

Since the early thirties there has been a continuous stream of literature relating steroids to hypertension. Perhaps recent experimental evidence of Tobian and Benoin

L. Tobian and J. T. Benoin, Circulation, 5:754,1952.

10
suggests the best interpretation to explain the apparent association between blood pressure level, salt retention, and DOCA effect. At autopsy they found hypertensive patients to have had a significantly increased sodium and water content of the psoas muscle and renal artery. It is their speculation that the same process occurring in the arterioles would narrow the lumina and therefore increase peripheral resistance. This experiment, too, has its faults, for it is well recognized that water content of extracellular space increases with age, and none of the patients coming to autopsy were listed according to age. Also, that DOCA acts as replacement therapy and produces experimental hypertension does not prove that it is a hormone of the adrenal cortex causing the basic malfunction in this state.

Adrenalectomy as a tool in the study of the
production of experimental hypertension

Another approach may be made to the role of the steroids in the genesis of hypertension. If these compounds play a critical role in the development of hypertension, then clinical observations of elevated blood pressure in cases of Addison's disease would not seem likely. Even so, a few papers have appeared reporting the observation of elevated blood pressures in cases of Addison's disease. However, in the patient reported by Loeb, the blood

R. F. Loeb, Bull. N. Y. Acad. Med., 18:263, 1942.

M. Cohen, Arch. Int. Med., 81:879, 1948.

pressure was only 160/100 on sodium chloride therapy alone. Nevertheless, this does not invalidate the classical picture of low blood pressure seen in this disease. The persistence of low blood pressure in this disease is as valid an argument in favor of an adrenal role in the regulation of normal pressure as the exceptional case of elevated pressure is against this regulation. The question, however, is whether or not the adrenal cortex is essential as part of the final common pathway in blood pressure regulation. It would seem from animal experimentation that less divergence of facts exists concerning the role of adrenalectomy in experimental hypertension than in the interpretations derived from these facts. Goldblatt was the first to

H. Goldblatt, Ann. Int. Med., 11:69, 1937.

show that adrenalectomy prevented the development of experimental renal hypertension by his described technique, and this has repeatedly been confirmed since. Blalock, Page and Remington were able to show that the lack of pressor effect is attributable to inability of terminal arterioles to respond to pressor agents after adrenalectomy is strengthened

A. Blalock and S. E. Levy, Ann. Surg., 106:826,1937.

I. H. Page, Am. J. Physiol., 122:352,1938.

J. W. Remington, W. D. Collings, H. W. Hays, W. M. Parkins and W. W. Swingle, Am. J. Physiol., 132:622,1941.

by the results of Zweifach and Shorr. They were able to

B. W. Zweifach and E. Shorr, First Clinical Acth. Conf., New York, The Belakiston Company, 1950, p. 288.

show that the mesenteric arterioles of adrenalectomized rats, as compared to controls, failed to show an increased threshold response after capping of kidneys to cause perinephritis. It is interesting that they report that in the absence of the vaso-excitor material ("VEM", now in disrepute) produced by the capped kidney, the threshold response to epinephrine of adrenalectomized rats maintained on salt was as good as the controls. Either adrenal cortical extracts of DOCA could be substituted adequately for the adrenal glands both in maintaining the threshold response and in the production of "VEM" by the kidney.

The difficulty of maintaining adrenalectomized animals in a normal physiological state without steroid replacement therapy has made it impossible to appraise the results fairly. Thus many criticisms have been leveled at the above results obtained by Zwiefach and Shorr.

Remington suggests that the mechanism may involve a

J. W. Remington, Am. J. Physiol., 165:306, 1951.

a metabolic failure of the sympathetic nervous system and thus account for the results of these two workers.

For practical purpose, it has been demonstrated by several workers that subtotal adrenalectomy is able to lower the blood pressure of severely hypertensive patients to normal levels.

D. M. Green, J. N. Nelson, G. A. Dodds and R. E. Smalley, J. A. M. A., 144:439, 1950.

S. F. Wilhelm and E. Dickler, J. A. M. A., 147:1235, 1951.

C. C. Wolfarth, W. A. Jeffers, F. D. W. Lukens, H. A. Zintel and J. H. Hofkenschiel, Ann. Int. Med., 35:8, 1951.

H. A. Zintel, C. C. Wolferth, W. A. Jeffers, J. H. Hofkenschiel and F. D. W. Lukens, Ann. Surg., 134:351, 1951.

These workers found that when sufficient amounts of the gland are removed (about 95%) the reduction in blood pressure is variable and depends upon the initial diastolic pressure prior to surgery. They feel that this strongly suggests that intact adrenals are not only essential for both

experimental and human hypertensive disease, but are necessary for normal blood pressure maintenance. Since it has been established beyond a doubt that the adrenal gland, as far as the cortex is concerned, is under the control of adrenocorticotrophic hormone of the anterior pituitary, it would seem that ACTH must play a role in hypertension. Therefore, it is not surprising that an elevation in blood pressure in normotensive and hypertensive patients has been observed following ACTH administration. The significance, however, is that the effect is not impressive except on prolonged treatment, in which case

G. A. Perera, Proc. First Cl. ACTH Conf., New York, The Blakiston Co., 1950.

R. G. Sprague, M. H. Power, H. L. Mason, A. Albert, D. R. Mathieson, P. S. Hench, E. C. Kendall, C. H. Slocumb and H. F. Holley, Arch. Int. Med. 85:199, 1950.

H. Dustan, A. C. Corcoran, R. D. Taylor and I. H. Page, Arch. Int. Med., 87:627, 1951.

other signs of hyperadrenocorticism also occur.

Nevertheless, the elevation appears on usual therapeutic doses and the stimulus has been assumed to be physiological. An adrenal steroid with pressor properties being secreted under the influence of ACTH would account for this effect. However, more recent methodological advances

in protein chemistry have enabled Reinhardt and Li to

W. O. Reinhardt, and C. H. Li, Proc. Soc. Exp. Biol. and Med., 76:836, 1951.

separate posterior lobe contaminants from purified ACTH preparations, thereby significantly reducing both anti-diuretic and pressor properties of ACTH. Thus one might conclude that the clinically seen pressor properties of ACTH may not be an adrenal effect. This finding would be in accord with the present concept that ACTH does not greatly stimulate salt retention. Recent studies have shown that aldosterone is synthesized only in the zona glomerulosa, and that this zone is relatively poorly stimulated by ACTH. Until recently, with the event of removal of the hypophysis and its inability to affect the secretory activities of this zone, it was believed this portion of the cortex to be autonomous in function. However, the recent work of the group at Western Reserve University has shown that control of aldosterone output lies in the

A. E. Neuman, E. S. Redgate and G. L. Farrell, Endocrinal., 63:723, 1958.

area of the diencephalon. Preliminary evidence indicates that the pineal gland may produce a substance, glomerulo-

trophic hormone, which selectively stimulates the outer zone of the adrenal gland to produce aldosterone. However, these arguments do not preclude the possibility of ACTH stimulating the remaining zones to produce increased amounts of steroids. Also, there is a possibility of increased amounts of steroids being produced in a form not usually found in normotensive patients. No experimental evidence for these assumptions has been available to date. However, if increased amounts of steroids with aberrant molecular structures should occur, these steroids may alter the normal pathway for metabolism and excretion.

The blood pressure response to cortisone and/or hydrocortisone depends apparently on the clinical state of the patient as much as it does on the pharmacologic action of the drug. Thus the literature presents many reports ranging from control of hypertension to production of malignant hypertension by these drugs. In general, however, the blood pressure effects are minimal and secondary to other major metabolic changes encountered when these drugs are administered. No apparent concrete effort has been made to classify these divergent effects; many more studies will be necessary. A typical case may be cited to demonstrate the results obtained. Shepard and Clausen treated a five and one half year old boy having adrogenital syndrome and a blood pressure of 200/100 mm Hg.

H. Shepard and S. W. Clausen, Am. J. Dis. Child.,
83:262,1952.

Daily cortisone acetate administration of 40 mgms was given for 13 days: following this period it was reduced to 10 mgms per day. It was found that on this treatment the blood pressure fell to normal ranges.

Much less dramatic, but similar directional changes have been reported by others. Shroeder observed the same

H. A. Schroeder, J. Clin. Invest., 29:262,1950.

results in a mildly hypertensive patient subsequently shown to have an adrenal tumor.

In contrast, severe and reportedly malignant hypertension has been seen to follow cortisone therapy in sub-acute disseminated lupus erythematosus and periarteritis nodosa.

B. I. Heller, W. E. Jacobson and J. F. Hammarsten, J. Lab. and Clin. Med., 37:133,1951.

T. Ehrenreich and E. V. Olmstead, A. M. A. Arch. Path., 52:145,1951.

In the report by Ehrenreich, the patient received 200 mgms

of cortisone in the first two days. Heller reports a slight lowering of blood pressure in acute stages of Lupus with a reversal of the effect as the disease progresses. This is an agreement with the conclusions of Perera that

G. A. Perera, Proc. Soc. Exper. Biol. and Med., 76:583, 1951.

the pressure is elevated by cortisone therapy in the presence of acute renal changes.

Urinary Steroid Excretion in Hypertension

Repeated analysis of urinary steroids in hypertension has not clarified the role of the adrenal. Most of the evidence has been negative. Recent studies have shown that urinary aldosterone excretion is elevated in

J. Genest, G. Lemieux, A. Davignon, E. Koiw, W. Nowaczynski and P. R. Steyermark, Proc. Soc. Exptl. Biol. and Med., 97:676, 1958.

J. Genest, E. Koiw, W. Nowaczynski and G. Leboeuf, J. Clin. Invest., 35:706, 1956.

hypertension, but this is probably a poor index of actual production. However, more rigidly controlled studies utilizing a battery of renal function tests and serum electrolytes are indicated if these reports are to be

J. W. Conn, Circulation, 17:743,1958.

substantiated. Independently, Albert, Morita and Iseri have

D. G. Albert, Y. Morita and L. T. Iseri, Circulation, 17:761,1958.

compared serum magnesium, sodium and potassium concentration in essential hypertensives and in normals. These workers claim the serum sodium to be significantly higher in the hypertensive patients (147.7 ± 5 as compared to 142 ± 3.0); from these values they argue in favor of an excessive adrenal cortical activity. Many of the early urinary studies which seemed promising, when hypertensive and normotensive urines were studied, must today by necessity be disregarded. To cite one such study let us review the work of Dobriner. This group reported finding delta 9-etiocholenolone in the urines of three of six patients with hypertension, in five of seven patients with Cushing syndrome, and only two of twenty-four normal subjects. In addition, other unidentified compounds, which did not occur in Cushing's syndrome, were found in five cases of essential hypertension and only as a rare trace in normal urines. Current chemical techniques have shown, at least as far as the delta 9-etiocholenolone is concerned, that

this and many other steroids may result as artifacts in the hydrolysis and extraction procedure. Therefore, one is justified in disregarding work utilizing these drastic techniques which resulted in laboratory artifacts. One study, due to the nature of the techniques used, should receive recognition. Corcoran and associates have ob-

A. C. Corcoran, I. H. Page and H. P. Dustan, J. Lab. and Clin. Med., 36:297, 1950.

served that certain cases of essential hypertension appeared to be different from the standpoint of normal urine study. They found increased excretion of formaldehydrogenic corticoids in 20 out of a total of 46 hypertensive patients, levels being consistently elevated in six of these. However, in the hypertensive group there was a consistent increase in the variability of the daily excretion of these compounds. In contrast to these findings, Daughaday and Tobian failed to confirm the results

W. H. Daughaday, H. Joffe and R. H. Williams, J. Clin. Endocrinal., 8:244, 1948.

L. J. Tobian, J. Clin. Endocrinal., 9:319, 1949.

of others when the urines of hypertensive and other types of patients were compared with normals. Perhaps these

results can be criticized due to the small number of cases studied.

Hetzel studied adrenal cortical function in various

B. S. Hetzel and D. C. Hine, J. Clin. Endo., 12:296, 1952.

clinical types of hypertension by urinary assay. Urinary glucocorticoids were studied by bioassays (at best a very poor assay) and 17 ketosteroids by a chemical technique. A total of 40 hypertensive men and women were studied in contrast to 21 normotensive patients. They conclude that no direct evidence existed, as far as they were able to determine by these urinary steroid methods of an increased adrenal cortical function in hypertension.

Adrenopathological Findings in Hypertension

Many causes of renal and cardio-vascular pathology have been reported in hypertension. An equal number of claims have been made for adrenal hypertrophy and adenomatous changes in hypertensive patients coming to autopsy. Shorr and co-workers have presented an excellent review of these

E. Shorr, Tr. Am. Physicians, 64:445, 1951.

findings. Nevertheless it remains a controversial point; the weight of evidence being in favor of a greater incidence

of such findings in hypertensive than in normotensive persons. Dawson, in an extensive review, studied the weight of 90 adrenals from normotensive patients, 90

P. Dawson, J. Path. Bact., 72:393, 1956.

adrenals from patients with essential hypertension and 44 adrenals from patients with renal hypertension. The average weight of the normal adrenals was reported as 11.2 grams while the adrenals in the cases of essential hypertension weighed 15.3 grams. In the group of renal hypertensives the adrenal weight was reported as 15.7 grams. The essential difference between the renal hypertensives and the essential hypertensive adrenals was due mainly to an increase in the zona reticularis in the patients with renal hypertension. The increased weight in the adrenals from the patients with essential hypertension was due primarily to an enlargement of the zona fasciculata which exhibited irregular cells with hyperplasia and vacuolated nuclei. The lipid concentration of this zone was also increased as demonstrated by histochemical techniques. He was not able to demonstrate any change in the zona glomerulosa in either form of hypertension. Since aldosterone is secreted by the zona glomerulosa and no changes were noted, it would appear that aldosterone was not significant in these patients. Of interest is the enlargement of the zona

fasciculata which is implicated in the production of 17-hydroxycorticoids. The increase in the zona reticularis in the renal hypertensives, due to its primary production of androgens, would seem to leave one at a loss as to an adequate explanation of this difference.

Peripheral and/or Adrenal Vein Blood Steroid

Concentration in Hypertension

Many plasma corticoid studies have been carried out to determine if a significant difference exists between the steroid content of the normotensive and the hypertensive patient's plasma. Most of these studies have yielded equivocal results. Tobian studied the plasma formalde-

L. J. Tobian and H. Joseph, J. Clin. Endo., 9:677, 1949.

hydrogenic corticoids in normo- and hypertensive blood. Plasma corticoids in 8 hypertensives and 7 normal patients were not different. Concurrent studies such as fasting blood sugar levels, plasma CO₂ combining power, plasma CO₂ to chloride ratio, all resulted in nonsignificant differences between the normotensive and the hypertensive group. Nelson studied a pool of normal blood by means of

D. H. Nelson, L. T. Samuels, D. G. Willardson and F. H. Taylor, J. Clin. Endo. 11:1021, 1951.

the Porter-Silber reagent. He concluded that, on the basis of the extinction coefficient, all the color was due to cortisol. In a study of 680 milliliters of pooled plasma, Blayliss and Steinbeck were able to demonstrate

R. I. S. Blayliss and A. W. Seinbeck, Biochem. J. 54:523,1953.

50 micrograms of 17-hydroxycorticoids by a Porter-Silber modification. However, by chromatography, only about one half (25 micrograms) was found to be 17 alpha-hydroxycorticosterone (cortisol). Other more polar compounds located by chromatography were found to correspond to tetrahydrocortisone and some other more polar compounds which were not identified. It is their conclusion, since they do not agree with Nelson, that their results may be due to the plasma they used being obtained from patients with hypertension.

In another study by Romanoff a total of 500 milliliters

J. Romanoff, Clin. Endo. and Met., 13:1546,1943.

of human adrenal vein plasma was collected from a hypertensive patient with metastatic carcinoma of the prostate. From this volume of blood they were able to isolate 4.5 mgms of crystalline hydrocortisone and 0.40 mgms of

crystalline corticosterone. Also, tetrahydrocortisone and tetrahydrohydrocortisone were found as more polar substances.

Just prior to and following this work, Perera and

G. A. Perera and K. L. Pines, Proc. Soc. Exptl. Biol. and Med., 71:443, 1949.

Pines, Woodbury, Rosenberg and Sayers and Selye suggested

D. M. Woodbury, C. A. Rosenberg, and G. Sayers, Fed. Proc. 9:139, 1950.

H. Selye, J. Clin. Invest. 29:844, 1950.

that decreased formation of glucocorticoids rather than overproduction of mineralocorticoids might be the essential feature of hypertension, since in the hypertensive dog, the pathological changes produced by administration of the mineralocorticoid DOCA, could be prevented by simultaneous treatment with cortisone.

However, several other workers believe that the distribution of salt and water in hypertension would support the concept of increased activity of mineralocorticoids.

D. M. Green, A. D. Johnson, W. C. Bridges and J. H. Lehman, Circulation, 9:416, 1954.

E. Braun-Menendez, CIBA Foundation Symposium on
Hypertension, Boston, Little, Brown and Co., 1954.

Touchstone collected and extracted human adrenal vein

J. C. Touchstone, Fed. Proc., 17:382, 1938.

blood from hypertensives. Normal human adrenal vein blood was used as a control. Following chromatography, he was able to demonstrate a significant difference between the steroid profile pattern in the normotensive and the hypertensive patient. The major difference noted was that of a decrease in the hydrocortisone area and increase in the more polar compounds (more polar than cortisol area) in the hypertensive patients. On further chromatography, this polar area could be resolved into several component areas. One of these areas was tentatively identified as 6-beta hydroxhydrocortisone. The remaining areas are as yet unidentified.

CHAPTER III

METHODOLOGY AND TECHNIQUES USED

Clinical Preparation of Dogs

Since the original publication by Goldblatt, describ-

H. Goldblatt, J. Lynch, R. F. Hanzal and W. W. Summer-
ville, J. Exper. Med., 59:347, 1934.

ing a surgical technique that results in the development of hypertension of renal origin, many workers have utilized this procedure for various types of studies dealing with a wide variety of unrelated modalities.

As originally described by Goldblatt, all operations were performed under aseptic conditions, with the animal under nembutal anesthesia. An abdominal incision was made and by retroperitoneal approach the main renal artery was identified. On this artery a special clamp was applied.

In this study, occasionally a modified technique was employed. This consisted of a two-stage operative preparation, utilizing a unilateral nephrectomy followed later with contra-lateral renal constriction by means of umbilical tape drawn in a "figure-eight" around the kidney, according to Grollman and Harrison.

A. Grollman and T. R. Harrison, Proc. Soc. Exp. Biol. and Med., 52:162, 1943.

Thus all the hypertensive dogs in this study were prepared by either of these two methods; this resulted in renal hemodynamic changes, producing the desired systemic arterial blood pressure increase.

Collection of Adrenal Vein Blood

The dog was anesthetized with nembutal given i. v. and the trachea was cannulated. An abdominal incision into the peritoneal cavity was made. Novocaine was injected at the operation sites in order to reduce epinephrine secretion. The adrenal vein was tied. The lumbo-adrenal vein was dissected free and cannulated with a small specially designed glass cannula. Coagulation was prevented by injection of heparin.

A donor dog was anesthetized with ether to avoid accumulation of nembutal in the experimental dog. Blood was transfused from the donor to the experimental dog to replace the blood removed via the adrenal vein. The transfusion volume was determined by the amount required to maintain a relatively stable blood pressure for periods up to eight hours (see Chart X). Blood pressure could be read directly on a mercury manometer which was connected to a femoral artery of the dog.

Blood was collected in tubes, kept chilled in cold water until it was centrifuged in the cold and the plasma withdrawn. The plasma was stored at -20°C . until extracted.

Concurrent Clinical Data

Following the death of the dog, the adrenals and the heart were removed and weighed. Gross pathological changes of the kidneys, adrenals and heart were recorded and sections were taken for microscopic studies.

To determine whether or not the prolonged experimental procedure was playing a role in the steroid profile being produced, possibly by anoxia as reported by Gann,

D. S. Gann, I. H. Mills and F. C. Bartter, The Endocrine Society Meeting, June 4-6, 1959, Atlantic City.

an occasion tube of blood was processed separately and the individual concentrations of steroids determined.

Steroid Laboratory Techniques Utilized

Introduction

The laboratory techniques utilized in the work-up of the samples will be described, each in turn, in the order of their utilization in the identification of the compounds under analysis. It should be realized that this chapter is designed to lend emphasis to the practical aspects of the extraction, separation, isolation, and analysis of the steroids. Consequently the theoretical aspects of the various techniques have been minimized. In doing so, it has been necessary to specify details of methods for analyzing adrenal steroid contents of blood, tissue, and urine, and to describe those methods which can be used routinely for this purpose. It is obvious that a full treatment of the various systems which would be necessary for this type of evaluation would require a separate thesis.

The procedures used in the early isolation and identification of steroids were adequate indeed for the excellent work which was carried out by many workers. However, such techniques did not lend themselves readily to analytical studies in which it is necessary to compare relative amounts of these individual steroids, at the microgram level, in various experimental groups. Thus

within the last 15 years methodological advances have now permitted the isolation and identification of steroids at the 1 to 2 microgram level.

General preparative techniques have been dealt with somewhat minimally; however, specific physical-organic techniques for the absolute identification of a crystal isolated by the above techniques have been described in detail.

Paper chromatography, in the final analysis, is rather an art than a science, thus, it is necessary to practice the techniques daily in order to familiarize oneself with the "idiosyncrasies" of the systems. An example cited by Axelrod is in order. Good resolution of

L. R. Axelrod, personal communications, 1957.

estrogen epimers was carried out at Rochester University, by the systems devised by Axelrod. However, poor resolution was obtained by Axelrod at Southwest Foundation in Texas. Subsequently, it was found that the developing solvent, methylcyclohexene, although reagent grade was used in both laboratories, was prepared at two separate chemical plants. Impurities, not sufficient to appear on the label, were found necessary for the eventual resolution of these epimers. Thus it was necessary to purchase the same reagent grade chemical from the chemical plant

manufacturing the solvent in the East, rather than the chemical plant manufacturing the solvent in the South.

Extraction

All solvents utilized in this study for the extraction of blood, urine, or tissue were reagent grade chemicals. Occasionally chemicals of other grades were obtained, however these were purified according to the procedures described for this specific purification by Weissberger and Proskauer. The general extraction method

A. Weissberger and E. Proskauer, Organic Solvents, London, Oxford Press, 1935.

described by Delsol was used. Previous work (Southwest

J. L. Delsol, Bull. Soc. Chem. Biol., 26:99, 1944.

Foundation for Research and Education) has shown that six times two volumes extraction of plasma resulted in a 96% recovery of added steroids. However, the most polar steroids studied there was cortisone.

Sacrifice, perhaps, of a better method of extraction was necessitated by the requirement for continuous monitoring of the fractions for the content of bioactive material.

The Delsol's extraction procedure was essentially as

following: Plasma or tissue ^{is} was extracted with Delsol's mixture (dimethoxymethane and methanol, four to one) using one volume of plasma to two volumes of Delsol's mixture.

K. Savard, P. K. Besch, S. Restivo and J. W. Goldzieher, Fed. Proc., 17:303, 1958.

This extraction is repeated for a total of six times. The one volume of plasma is poured into two volumes of Delsol's and mixed well. This is allowed to stand for ten minutes, then filtered through Whatman No. 54 filter paper, in a Buchner funnel. The residue from the filter paper is then extracted five times more. The filtrates are combined and contain the total lipids. This combined extract can be reduced to one half its volume on a flash evaporation before the next step. The lipid extract from the above Delsol's mixture is partitioned with the following solvent mixture: diethyl ether, 2.5 volumes; isopropyl ether, 1 volume; and water, 1 volume. This is shaken in a separatory funnel and the water soluble fraction allowed to settle into the aqueous phase. The aqueous phase is discarded. The water wash is again repeated with 1 volume, shaken and the water phase again discarded. Removal of the ~~ether~~ phase is carried out with a flash evaporator, keeping the temperature below 50° C.

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The resulting oily residue is refrigerated at -20° C. until used. When the residue is removed from the icebox it is dissolved in ethyl acetate and partitioned against water. The water is discarded and the ethyl acetate is dried for further work. Certain notes of caution should be pointed out. During the extraction procedure there are many steps where emulsions are formed. These emulsions contain large quantities of steroids and therefore must not be discarded. Generally, two methods of breaking emulsions

K. Savard, personal conferences, 1958, 1960.

have been employed. One by centrifugation and two by cooling and then dropping the emulsion from a height of three feet.

In order to study the specific partition of the very polar compounds encountered later in this study, the following experiment was carried out. Known quantities of hydrocortisone, tetrahydrohydrocortisone, 20-beta hydroxy-tetrahydrocortisone, and 20-beta hydroxytetrahydrohydrocortisone were dissolved in plasma and separated into three representative fractions. Fraction 1 was extracted with methylene dichloride, fraction two extracted with ethyl acetate and fraction three was extracted with Del-solutions. The extracts were concentrated and chromatographed. The four areas representing the above steroids

were located and eluted from the paper. Quantitative extinction coefficient studies were carried out in sulphuric acid and the percent recovery of each compound plotted in Table 1.

Counter Current Distribution Studies

Large volumes of relatively closely related steroids can be resolved by the general technique of counter current distribution as described by Craig and others. The

L. C. Craig, J. Biol. Chem., 155:519,1944.

L. C. Craig and T. P. King, Fed. Proc. 17:1126,1958.

H. Carstensen, Acta. Soc. Med. Upsal., 61:26,1956.

L. L. Engle, J. Alexander, P. Carter, J. Elliott and M. Webster, Anal. Chem., 26:639,1954.

A. A. Levi, Biochem. J., 69:516,1958.

use of a 30 tube counter current distribution apparatus with a 40/40 ml phase system has been employed. Large volumes of the residue from Delsol's extraction of adrenal vein plasma has been distributed between petroleum ether and water. By means of bioassay after the appropriate combination of tubes, the activity was found in the water phase. Chart I represents the results of a typical distribution in graphic form. It is easily seen, from the BTZ extinction values and the chromatography, that the more polar compounds were partitioned into the water phase.

Table 1. Per Cent Extracted by Different Solvents.

	MECl ₂	ETAC	Delsol's	
			water	organic
F	91	93	8	90
THF	85	83	---	80
20b-OH-THE	61	65	---	59
20b-OH-THF	55	59	---	49

CHART 1

BTZ Reduction per Tube of CCD

Typical partition coefficients as reported by Silber and Porter are summarized in Table 2.

R. H. Silber and C. C. Porter, Methods of Biochemical Analysis, 4:139, New York, Interscience Press, 1957.

This table has been designed to show the partition of a variety of steroids from a water phase (urine, blood, etc.) into six different organic solvents of varying polarity.

A non-polar solvent such as PE can be safely used to wash aqueous samples containing cortical steroids. Such solvents extract not only lipids but also certain steroids which do not bear the dihydroxy acetone side chain. CCl_4 extracts very little dihydroxyacetone side chain steroids from aqueous solution but is a fairly good solvent for the extraction of less polar corticoids, estrogens, and certain other steroids. MECl_2 has solvent properties practically identical to those of CHCl_3 ; however, it has the advantage over CHCl_3 in that it shows no tendency to decompose to yield phosgene and HCl .

Certain 21-esters of dihydroxyacetone side chain steroids, e.g., t-butyl acetate of E & F, can be extracted from aqueous solutions with CCl_4 , whereas the parent free alcohols cannot. Thus, the esters and alcohols can be separated from one another and their concentrations

Table 2. Partition of Steroids between HOH and Organic Solvents

	CHCl ₃	ETAC	Benzene	CCl ₄	PE	MECl ₂
E	0.027 ^a	0.057	0.9	30	500	0.033
F	0.14	0.078	2.7	120	500	0.09
THE	0.12	0.048	3.3	75	500	0.065
THF	0.57	0.073	7.9	250	500	0.4
CompB	0.005	0.055	0.08	0.75	190	0.0065
Testo	0.01	0.01	0.01	0.03	0.036	0.01
Prog	0.01	0.01	0.01	0.02	0.01	0.01

a The smaller the number, the more solubility preference for the organic solvent.

independently estimated.

The same note of caution regarding the formation of emulsions are again applicable to countercurrent distribution. Since this is essentially the distribution of a solid between two organic solvents, the same physical forces apply that are applicable to the paper and column chromatography. It should be pointed out that all solvents, before their use in the countercurrent distribution, should be equilibrated with each other, and once the distribution begins the temperature should remain constant. With this in mind, we have found it useful to equilibrate our solvents in the walk-in cold room (5° C.) and subsequently to run the countercurrent distribution in the same room. Since most runs are at constant room temperature it is recognized, therefore, that results obtained by us are not comparable to those obtained by others. This means that our partition ratio values are radically different from those reported by others in the literature. However ultimately, our results are comparable to theirs.

Column Chromatography

When large amounts of steroid mixtures are to be separated, column chromatography has been found to be most useful. All the column chromatography utilized in this work was partition chromatography. In the synthesis work, reaction mixtures were extracted and found to contain several steroids by paper chromatography. When 300 to

500 mgms of the steroids is being made, it would be a laborious job to resolve this amount by paper chromatography. Thus the use of column chromatography has played an important role in the separation of epimers. (See Synthesis Section.) Once the identification of the compounds had been established by paper chromatography, the proper solvent for the elution of the column can be determined. Thus, the solvent necessary to effect the proper fractionation can be employed for the resolution of these large quantities. It has been found that 1.3 grams of silicic acid can normally handle up to 100 mgms of steroid. Thus if the residue consists of 100 mgms, or multiples thereof, 1.3 grams of silicic acid or multiples thereof are employed as stationary support for the column chromatography. No stationary phase as such is utilized in this type of chromatography.

To assure reproducible results it has been found that columns packed by gravity consistently result in uniform fractionation. Thus in the preparation of the column, silicic acid is suspended in the least polar solvent to be employed and slurried into the column. After the silicic acid has been packed by gravity, the most polar solvent is then added. The addition of the most polar solvent is used to eliminate any possible contaminants from the silicic acid. After the appropriate volume of the most polar phase has passed through the column, the column is

then returned to the least polar solvent. The sample can be placed directly on the silicic acid or can be spotted on a piece of filter-paper disc cut to the size of the inside diameter of the column and placed on top of the silicic acid. The appropriate solvent for elution is then added and the column is placed above a constant fraction collecting apparatus. A reservoir containing the appropriate solution is then added and fractions collected as necessary to affect the proper resolution of the added material.

For volumes such as 50 ccs of plasma, it has been found helpful to use column chromatography instead of countercurrent distribution, to resolve the very polar lipids from the steroid material. A typical column employing two grams of silicic acid will support approximately 450 mgms of extract residue. This residue is placed on top of the column prepared in benzene, and 5% ether in benzene is added. By this elution, 5% ether in benzene, all of the steroids less polar than corticosterone are removed. The remaining steroid fractions more polar than corticosterone can then be removed in toto, by elution with ethyl acetate. Each fraction thus obtained from the column is dried on a steam water bath and chromatographed on paper. By this method, in this laboratory, a recovery of 91% of added hydrocortizone has been obtained. Specific application for the resolution of alpha and beta epimers of a

parent compound are reported in the individual sections in the synthesis chapter.

A note of caution should be added at this point regarding polarity of steroids. If it is found that the steroids by paper chromatography run on the solvent front, it is not necessarily true that the same solvent polarity will affect the elution of this compound from the silicic acid column. It has been our usual policy, with highly successful results, to increase the polarity of the solvent, thus achieving fractionation of the steroid from the column. Steroidal ketal formation carried out in ethylene or propylene glycol may result in residues containing large quantities of the glycol. In the presence of this glycol, it is impossible to achieve crystallization of the compound. Many differential methods of solvent extraction have resulted in the continued presence of this glycol in the final residue after evaporation. Thus, it has been found helpful to add to this oily residue equal parts of charcoal and silicic acid. This mixture of residue, charcoal and silicic acid are thoroughly mixed in a mortar and pestel and quantitatively transferred with the aid of hot acetone to a glass column. The steroid can be eluded by successive additions of hot acetone and collecting the runoff fraction. No quantitative recovery studies have been done, since the method was utilized to separate large quantities (300 to 500 mgms

of steroid mixtures).

The use of alumina columns is often warranted when very large quantities are being processed for separation as the amount of substance for the column per quantity of steroid is a smaller ratio than with other types of column material. Thus the volume of solvents for elution is far less.

In general, a word of caution, when steroids of the dihydroxyacetone side chain structures are placed on alumina columns. There is often noted a rearrangement of the steroidal structure. However, if the acetate derivative is utilized, this rearrangement is limited to a very small percentage of the total steroid chromatographed. This has been adequately demonstrated in our laboratory by the following experiment. Hydrocortisone, free alcohol, has been placed on alumina and the column developed. Comparable quantities of hydrocortisone acetate were also placed on a second alumina column and eluded. Paper chromatography of the two elution products resulted in four spots for the free alcohol and a single spot for the acetate derivative. Of the four spots for the free alcohol, one was less polar, one ran with the polarity of hydrocortisone and two were more polar. Chemical studies of the least polar compound, failed to reveal a dihydroxyacetone type of side chain. Also negative result for a 17 ketogenic steroid was obtained. Thus it would seem

that the alumina chromatography of the free alcohol of hydrocortisone, as far as the least polar compound is concerned, resulted in the selective removal of the 17 alpha-hydroxy group. The two more polar compounds observed were not chemically characterized.

Analytical Paper Chromatography

As will be apparent from the following discussion, paper chromatography is a basic technique for evaluating the progress of steroid separation by any or all of the methods utilized throughout this entire work. It is invaluable also in synthesis as well as in the study of derivative formation. It should be fully realized that personal choice determines the exact combination of chromatographic techniques to be used in resolving a specific mixture. From laboratory to laboratory, the same mixture may be separated by different procedures, yet ultimately the same results are obtained.

Primarily the paper chromatographic techniques and systems utilized were initiated by Zaffaroni, Axelrod,

A. Zaffaroni, R. B. Burton and E. H. Keutmann, J. Biol. Chem., 177:109,1949.

L. R. Axelrod, J. Biol. Chem., 205:173,1953.

and Nowaczynski. They can be adapted to investigation of almost any steroids.

W. J. Nowaczynski and E. Koiw, J. Lab. and Clin. Med.
49:815, 1957.

The ratio of the distance traveled by a compound to that traveled by a solvent front (the R_f value) is a characteristic of the substance under the prevailing conditions and is gradually being accepted as a reproducible physical constant. If the R_f value is extremely low, as in the case of the varyd polar steroids, this term is not utilisable because the solvent front has long transversed the end of the paper before the steroids have begun to move. Thus the ratio of the distance traveled by a varyd polar steroid to that of the solvent front has posed a problem in nomenclature. Various abbreviations have been suggested for this calculated value. In this laboratory, the designation R, with a subscript abbreviation of the standard used, means the ratio of the mobility of the unknown steroid to that of a specific standard reference substance. Thus any specific steroid can be designated more or less polar than the standard reference compound and the R values calculated. Therefore, it can be stated that the R value of a compound relates the polarity of the unknown to a known compound. Usually, the mobility of a compound is inversely related to the polarity of the compound. In other words, the more oxygen functions present,

the more polar and thus the more slowly moving, the greater the polarity of the solvent required to move the compound in question.

Since chromatography is a technique of separation, it is then worth considering, even briefly, the forces which bring about this separation. The main factors involved are those of partition between the solvent traveling along the paper (mobile phase) and the compound held stationary in the cellulose (stationary phase), adsorption (hydrogen bonding plus Van der Waal's forces, etc.) onto the cellulose of the paper, ion exchange on the carboxyl and possibly the hydrogen groups of the cellulose. Until recently it was thought that partition was the only force affecting separation and therefore elaborate effects were made to obtain conditions of thermodynamic equilibrium. Alternately, of course, the chief criterion for analytical work is the reproducibility of results and not necessarily the simulation of theoretical conditions. Zaffaroni, Axelrod, Hofmann and Standinger, and Savard

A. Zaffaroni, J. Biol. Chem., 188:763, 1951.

L. R. Axelrod, J. Biol. Chem., 205:173, 1953.

H. Hofmann and H. J. Standinger, Naturwissenschaften, 38:213, 1951.

H. Hofmann and H. J. Standinger, Biochem. Z., 332:230, 1951.

have accurately related the mobility rates of steroids

K. Savard, J. Biol. Chem., 202:457,1953.

in various systems and provide extensive references and detailed information. These authors have considered the effects of temperature, hydrostatic pressure, solvent vapor saturation of the chromatographic chamber, and other critical physical forces to the development of the differential migration of steroids on the paper chromatogram.

Materials Utilized

Control of temperature

Essentially three ranges of temperature were utilized for the development of the chromatograms throughout this study. 1. Above room temperature (33° centigrade) and 2. below room temperature (19°) and 3. ambient room temperatures.

Large insulated boxes were constructed similar to incubators. Each box housed four large chromatographic chambers. A constant temperature of 33° was maintained throughout the box, thus throughout the chromatographic chamber, by means of an external heated water bath connected to the box by a pumping apparatus. Within the box, heat from coils was circulated by a fan. Thermostatic control was maintained within the box. The below room temperature boxes (19° centigrade) were of a similar

construction. By means of an ammonium refrigerant plant, ice was frozen around external coils in a large water bath. By the same pumping and circulation system as described above, the temperature was maintained in the 19° boxes by thermostatic setting. Thus by raising the temperature above that of the room and maintaining it constant, it was possible to increase the mobility of certain varyd polar steroids by a factor of almost 2. This increased temperature was utilized for chromatograms running from one day to seven days. Likewise, by use of lower than room temperature (19°), better resolution was obtained between closely related steroids. These temperatures were also used for long-term chromatography. Short run chromatograms, from several minutes to several hours, were run in chambers at room temperature. Such compounds as $C_{19}O_2$ $C_{19}O_3$'s, and the vary unpolar pregnenes were developed by short term runs.

Chromatographic chambers

Two sizes of chromatographic chambers were used. Large pyrex chromatographic chambers, 12 x 24 inches, were extensively used in the temperature controlled chambers. These chambers contained a chromatocollar, which is a shelf or ledge-like apparatus so constructed as to hold stainless steel pans, and were closed by means of a round glass plate with a starch jelly prepared in this laboratory. The smaller chambers, 12 x 18 inches, used a stand-

type apparatus to hold one stainless steel pan. Each 50
chamber was lined with Whatman No. 3MM filter paper. On
the bottom of each chamber was placed two to three inches
of the developing solvent. Thus by means of the increas-
ed surface area, wick effect, equilibration of the cham-
bers was extremely rapid. Thus by using the large chambers
it was possible to simultaneously run an experimental
chromatogram and its normal control chromatogram.

Spotting board and micropipets

A chromatographic spotting board was developed in
this laboratory which essentially consisted of two lucite
pieces of plastic hinged at one end. At the opposite end
an inch wide hole ran almost the entire width of the two
pieces of lucite plastic. Thus it was possible to place
the chromatogram to be spotted between the two pieces of
lucite, with only a small area exposed, and spot samples
which often contained a great deal of lipid, yet prevent
drying of the stationary phase on the chromatogram.

Commercially available micropipets were used ranging from
one lambda to 500 lambda. These were used for placing
known amounts of standard compounds on the side limb
(reference strip). Micro transfer pipets, for sample
spotting, were prepared in this laboratory.

Size of chromatogram

All of the 2,000 plus chromatograms run in this study
were run on Whatman No. 1 filter paper. The size of the

chromatogram varies with the load applied. That is, normally a 16 centimeter wide chromatogram can hold up to 30 to 50 milligrams of sample. The final critical factor is that following development the resolved steroid shall not be concentrated more than 1 milligram per square centimeter. Above this concentration resolution is lost and trailing results.

A word of caution should be added at this point. It has been observed that the rate of mobility of a steroid is also dependent upon the width of the paper. Thus standards run on a narrow side limb may have migrated further than the unknowns on the wider paper.

Preparation of the Paper Chromatogram

Although special Whatman No. 1, called chromatographic filter paper, was used throughout the study, it was found necessary to preclean the paper before use. Oil absorbed from the rolling and packaging mechanisms was absorbed onto the paper. Thus it was found necessary to place the chromatographic paper in a clean chamber and allow a 24-hour petroleum-ether run. The residual petroleum-ether in the stainless steel pan was replaced with 95% alcohol and allowed a 24-hour run. Following these two runs the paper was removed and dried in an oven and stored over CaCl_2 in a plastic container. Before each individual sheet was used in chromatography it was scanned with ultraviolet light. If any of the sheets were found to

contain UV absorbing or UV fluorescent areas, they were 52
discarded.

The stationary phase of the chromatogram was applied by dipping the chromatogram through the proper solution. The chromatograms were then dried by placing between two clean pieces of filter paper and blotting. The stationary phases consisted of one of the following: propylene glycol and methanol, 1 to 1; formamide and methanol, 1 to 1; or ethylene glycol and methanol, 1 to 1. On the head of each chromatogram prior to dipping, was placed the number of that paper along with the system of development followed by the hours of the chromatographic run. Following the dipping in the stationary phase and the subsequent blotting, the paper chromatogram was placed in the spotting board.

The sample to be chromatographed was applied by means of micro-transfer pipetes. Two different types of application are used depending on the size of the sample. For larger samples, the sample is dissolved in equal parts of chloroform:methanol and streaked across the paper in fine lines until successive applications overlap. After each streaking application of the sample, the organic solvent is dried by means of a fine stream of nitrogen. If the sample to be applied was very small, the spotting technique is used. This consisted of applying the sample in a confined circular area, so common in chromatography of other types of material.

A word of caution should be added at this point regarding the application by means of streaking the sample across the paper. If the material applied, on the starting line, is allowed to flow to the very edge of the paper on either side, the sample by capillary action will become concentrated on the very edge of the paper. Thus when the chromatogram is subsequently developed, in the mobile phase, the greater concentration at the edge of the paper does not move with the same mobility as an area in the center of the paper. Therefore, the resolved steroid will not have an even front but will appear in the form of a "U". As pointed out above, standards run on a side-limb must be placed on a width comparable to the chromatogram if relative mobility interpretations are to be made.

Developing the Paper Chromatogram

All chromatograms in this series were by the descending technique. Except when specific requirements prevented its use, Durchlout, or run off, chromatography was used throughout the series. Classically, mobile phase solvents used for chromatography are prepared by shaking one organic solvent with a second organic solvent, the stationary phase, in a separatory funnel, separating the phases, and using the proper layer as the mobile phase. Thus, no equilibration effects take place between the stationary phase of the paper and the mobile phase being used for the development. The usual mobile phases used

throughout this study are toluene equilibrated with propylene glycol (T / PG), chloroform with formamide (C / F), and methylcyclohexene equilibrated with propylene glycol (M / PG). Other specific mobile phases were used as the demand arose. It has been observed that the volume of the mobile phase placed in the stainless steel pan determines, in part, the speed of the chromatogram. Therefore, 300 cc. was the standard amount used throughout the entire study. If the chromatogram was to be run longer than three days, as for the very polar steroids, the addition of 50 ccs. of the mobile phase was necessary after each three day period the chromatogram was being developed, in order to maintain a relatively constant flow.

A run-off beaker was placed beneath each chromatogram so that the mobile phase containing the steroids less polar than those remaining on the paper, can be collected. This beaker was given the same coding as the head of the chromatogram. At the appropriate length of development of the chromatogram the paper was removed and dried.

Drying was carried out by placing the chromatogram in a chamber so constructed to allow the papers to be hung by the head. Gentle heat was applied by infra-red bulbs containing no ultraviolet light as UV light will cause structural rearrangements. A suction fan at one end of the chamber permitted a gentle movement of air across the papers. Complete drying is essential.

Paper chromatography is a technique for effecting the separation of closely related steroids but, as the majority of compounds studied are colorless and so not visible on the paper, the problem of locating them still remains a major problem. The most widely used method is that of applying to the dried chromatogram a chemical reagent which will react with some or all of the compounds of interest to produce some visible color. In steroid chromatography this is generally done by dipping the chromatogram through a bath of the reagent followed by some appropriate treatment such as allowing to dry in air or heating until colors appear. The location reagent may be a general one, that is one that will react to locate all the steroids of interest, or a more specific one, that is one which reacts with only one specific steroid in the original mixture.

Evaluation of the resolved paper chromatogram

Analysis of the zone or area

Ultraviolet Absorption (UV). -- The property of UV absorption in the 240 mu region, which is possessed by all steroids containing an alpha, beta-unsaturated ketone, can be used to great advantage in the visual evaluation of resolved paper chromatograms. This method coupled with reagents affords a means of locating the steroids on the paper. The Mineralite UV Short Wave lamp has been found to be satisfactory for this purpose.

Chemical spot tests. -- Corroborative data pertaining to further identification of these isolated steroids can be obtained by subjecting small strips, 0.5 cm wide or less and as long as the entire chromatogram, to chemical tests carried out directly on the paper strip by dipping the strip through the reagent in a small evaporating dish. Each reagent used takes advantage of the molecular structures of the compounds under consideration. The usual chemical tests employed and steroids they elucidate are given in tabular form in Table 3 and Table 4 respectively.

Side chain identification by micro-organic analysis. -- This method has been employed by Axelrod. The sensitivity

L. R. Axelrod, Anal. Chem. 27:1308, 1955.

of the method is at the 25 ugm range. With this method, one can tentatively identify any of the nine side chain structures presented in Chart II. The scheme for this analysis is presented in flowsheet form in Chart III and IV. Again the strips are dipped through the reagents. The reagents designated as A through H in Charts III and IV are prepared as shown in Table 5. A complete discussion of the individual mechanisms of reaction and details of the analytic procedure are to be found in the paper by Axelrod.

Elution of the zone from the paper

After an area or zone has been located on the dry

Chart II. Side chains of steroids at carbon-17 detected by appropriately applied tests as outlined in Charts 3 and 4.

Chart III. Micro-organic Side Chain Analysis, A.

Chart IV. Micro-organic Side Chain Analysis, B.

Table 3. Reagents Used For Identification of Steroids

Reagent	Composition	Concentrations
TPTZ	Triphenyltetrazolium chloride	Stock sol of 0.2% TPTZ in HOH; add 2 pts TPTZ to 1 pt of 10% NaOH
BTZ	Blue tetrazolium chloride	500 mg% sol of BTZ. 20% NaOH in 60% MEOH. Mix 1:3 pts.
DNPH	2,4-dinitrophenylhydrazine	0.15 gm of DNPH in 9 N HCL
I in H ^I	I ₂ in Hydriodic Acid	Hydriodic acid saturated with I ₂ . Mix 1 pt. with 9 pts. of HOH.
SbCl ₅	Antimony pentachloride	1 pt. of SbCl ₅ in 2 pts. CHCl ₃ .
Na ^I	Sodium Iodide	10% Na ^I in aq-60% Acetone mixed with 24 N H ₂ SO ₄ in equal pts.
Ac-ETOH	Sulphuric acid	24 N H ₂ SO ₄ made in 95% ETOH.
Zimm	m-Dinitrobenzene	1% DNb in MEOH and 15% aq-KOH mixed 2 pts of reagent to 1 pt base.
Vanillin	Vanillin	0.25 gm vanillin in 100 ml of 85% phosphoric acid.
Soda-UV	sodium hydroxide	10% NaOH in 60% MEOH
Blue-UV	Trichloroacetic acid	aq-25% TCA
Tollens	Silver nitrate	2 pts 0.1 N AgNO ₃ to 1 pt 10% NaOH.

Table 4 - Steroid Color Reactions

Steroid	TPTZ	BTZ	DNPH	I in H ^I	SbCl ₅	NaI	Ac-ETOH	ZIMM	Vanillin
1'	r*	bl	or	--	or-br	-	-	-	-
2	r	bl	or	dr. bl	-	-	-	-	-
3	r	bl	or	or-y	or-br	-	-	-	-
4	r	bl	or	or-y	lt. br	-	-	-	-
5	r	bl	or	y-br	lt. br	bl	-	--	-
6	-	bl	or	y-br	lt. br	lt.y	-	-	-
7	-	p-bl	-	or-br	r-br-p	l-y	or-pi	p	-
8	-	p-bl	or	y-br	br	br	-	p	-
9	-	bl	or	-	lt.y-br	-	-	-	or-br
10	-	bl	or	g-bl	-	l-y	-	-	-
11	-	p-bl	or	y-br-bl	lt.y-br	l-y	-	-	-
12	-	p-bl	or	y	-	dk.r-br	-	-	-
13	r	bl	-	lt.or	br	-	-	-	-
14	r	bl	-	lt.or	-	-	-	-	-

Table 4 - continued

Steroid	TPTZ	BTZ	DNPH	I in H ^I	SbCl ₅	NaI	Ac-ETOH	ZIMM	Vanillin
15	r	bl	or	-	or-g	-	-	-	-
16	-	-	or	-	or-br	-	-	-	-
17	-	-	-	-	or-br	-	-	-	-
18	-	-	-	bl	br	-	-	-	-
19	-	-	-	lt.y-br	br	-	-	-	-
20	-	-	-	lt.or-br	br	-	or-pi	-	-
21	-	-	-	gr-bl	br	-	-	-	-
22	-	p-bl	y	or-br	-	lt.l-y	-	-	-
23	-	p-bl	y	lt.y-br	-	-	-	-	-
24	-	-	-	lt.y-or	dk.p	-	or-pi	-	-
25	-	-	-	lt.y-or	br	-	or-pi	-	-
26	-	-	-	lt.y-br	br	-	-	-	-
27	-	pi-bl	-	r-br-bl	br	-	lt.pi	p	-
28	-	pi-bl	or	br	lt.y-br	-	-	-	-

Table 4 - continued

Steroid	TPTZ	BTZ	DNPH	I in H ^I	SbCl ₅	NaI	Ac-ETOH	ZIMM	Vanillin
29	-	p-bl	y	or-br	--	lt.l-y	-	-	-
30	-	-	-	-	lt.y-br	-	pi	-	-

*Key

r - red

br - brown

g - green

bl - blue

y - yellow

gr - grey

p - purple

pi - pink

l - lemon

lt.- light

or- orange

dr.- dark

† See following page for key to steroid numbers.

Key to compounds 1-30 appearing in Chart II

<u>Compound</u>	<u>Relative Polarity</u>
1) Hydrocortisone	$C_{21}O_5$
2) Cortisone	
3) Corticosterone	$C_{21}O_4$
4) Dehydrocorticosterone	
5) Desoxycorticosterone	$C_{21}O_3$
6) Progesterone	$C_{21}O_2$
7) Δ^5 -Androstene-3 α -ol-17-one	
8) Δ^4 -Androstene-3,17-dione	$C_{19}O_2$
9) 11-Desoxy-17-Hydroxycorticosterone	$C_{21}O_4$
10) 17 α -Hydroxyprogesterone	$C_{21}O_3$
11) Testosterone	$C_{19}O_2$
12) 9 α -Br-11-Ketoprogesterone	$C_{21}O_3$ Br
13) Tetrahydro-hydrocortisone	
14) Tetrahydro-cortisone	$C_{21}O_5$
15) 9 α -F-Hydrocortisone	$C_{21}O_5$ F
16) 11 β -Hydroxyprogesterone	$C_{21}O_3$
17) Pregnan-3 α ,20 α -diol	
18) Allopregnan-3 β ,20 α -diol	
19) Allopregnan-3 β ,20 β -diol	
20) Δ^5 -Pregnen-3 β -ol-20-one	$C_{21}O_2$
21) Allopregnan-3 β -ol-20-one	
22) Pregnan-3,20-dione	
23) Allopregnan-3,20-dione	
24) Methylandrostene-3 β ,17 β -diol	$C_{20}O_2$
25) Androstan-3 α ,17 α -diol	
26) Androsterone (Androstan-3 α -ol-17-one)	
27) Epiandrosterone (Androstan-3 β -ol-17-one)	
28) Androstane-17 β -ol-3-one	$C_{19}O_2$
29) Androstane-3,17-dione	
30) Estriol	$C_{18}O_3$

<u>Reagent</u>	<u>Composition</u>	<u>Concentration</u>
A	Periodic acid	3% in MEQH:HOH 1:1 sol.
B	Chromic acid	0.4% in 90% HAc
C	Lead tetraacetate	sat. in HAc 1:19 vols.
D	Chromic trioxide	0.2% in acetic anhydride
E	Aluminum isopropoxide	1% in 1:1 toluene- cyclohexanone
F	Lithium aluminum hydride	2% in ether
G	Triphenyltetrazolium chloride	0.2% in 3.5 N NaOH
H	Dinitrobenzene	1% in KOH (15%) 2:1

Table 5. Reagents used in the Axelrod micro-organic analysis of the slide chain of steroids.

chromatogram by UV light and reagents, the area is marked with a hard lead pencil. This area is then cut from the paper and cut into squares no larger than 0.3 to 0.4 cms. The paper squares are then covered with a 1:1 mixture of Chloroform:MEOH and allowed to stand for 20 minutes with intermittent swirling. The eluting fluid is then poured into a sintered glass fitted funnel and vacuum is applied below. All of the squares are transferred to the funnel and the all remaining solution pressed from them by means of a rod with a flattened enlarged end. The squares are again returned to the elution flask and the entire procedure repeated twice more. It has been found when eluting very polar steroids from paper, it is well to raise the temperature to 45° C. and follow the normal elution with one elution of Acetone or ETAC. This, raising the temperature and the additional solvent, increases the recovery of these polar steroids to that of the usual less polar steroid. The combined elute is then taken to dryness at less than 45° and then transferred to a small test tube until used. By use of extinction coefficients of the compounds in MEOH, this type of elution affords a recovery of 96 - 102%. Unfortunately, colloidal size particles of the cellulose from the paper are also obtained. Thus before spectral analysis is carried out, it is best to attempt crystallization of the eluted compound. This can be achieved by a classified method according to Axelrod. By this method, quantities as

L. R. Axelrod, personal conference, 1959.

small as 250 ug can be crystallized. Typical crystallization properties are presented in Table 6.

A second method, according to Axelrod, can be utilized to remove the cellulose from the eluted sample. This is carried out by applying the eluted sample to a thoroughly clean piece of paper that has been run as a blank chromatogram is then placed in MEOH and the sample allowed to run off the paper into a beaker. Since cellulose has an affinity for cellulose, the colloidal particles do not flow from the paper. After this method of eliminating the impurities, the sample can be used directly for spectral studies. Extinction coefficients of the compounds in MEOH have shown recoveries to approximate 100% by this latter method.

Physicochemical Methods

Spectra in MEOH and ETOH. -- Identifying spectral peaks characteristic of specialized structural arrangements can be carried out in MEOH and ETOH. Some characteristic peaks and structures observed are as follows: Δ ⁴⁻³ ketone between 238 and 242 mu depending on the structure at C-11, Δ ^{4-3,6} diketone at 243 mu and Δ ⁴⁻³ ketone, 6b-ol at 235.5 mu. This subject is adequately covered by Dorfman.

Table 6. Crystallization Data.

<u>Steroid</u>	<u>Crystalline form</u>	<u>Recommended Solvents</u>	<u>Acetate</u>
Adrenosterone	Needles	Alcohol	-----
Allopregnan-3a,20a- diol	Needles	Alcohol	-----
Allopregnan-3a,20b- diol		Acetone	-----
Allopregnan-3b,20a- diol	Leaflets	dil. Alcohol	-----
Allopregnan-3b,20b- diol		Alcohol	-----
Allopregnan-3b,17a- diol-20-one	Platelets	Alcohol	-----
Allopregnan-3, 20- dione	Thin Plates	Alcohol + Ether	-----
Allopregnan-3a-ol- 20-one		dil. Alcohol	-----
Allopregnan-20a-ol- 3-one		Alcohol	-----
Allopregnan-3b-ol- 20-one	Plates	Alcohol	-----
Allopregnan-20b-ol- 3-one		dil. Alcohol	-----
Allopregnan-3b,11b, 17a,20b,21-pentol	Leaflets	Alcohol	Acetone
Allopregnan-3b,17a, 20b,21-tetrol	Leaflets	Dil. Methanol	Rods from Ether + Pentane
Allopregnan-3a,11b, 17a,21-tetrol-20- one	Needles	Alcohol	-----

Table 6 Continued

Allopregnan-3b,17a, 20b-triol	Rhomb. needles	dil. Acetone	Acetone
Allopregnan-3b,17a, 21-triol-11,20- dione		Alcohol	-----
Androstane	Leaflets	Acetone+ Methanol	-----
Androsterone		Acetone + Ether	Ether
Anhydrohydroxy- Progesterone		Ethyl Acetate	-----
Corticosterone	Trigonal plates	Acetone	Needles from Acetone
Cortisone	Rhombohedral platelets	95% Alcohol	Flat needles from Acetone
11-DehydroCorti- costerone	Lg. prisms	Aq. acetone	Needles from alcohol
Dehydroepiandro- sterone	Needles and Leaflets	Alcohol	-----
Desoxycorti- costerone	Plates	Ether	Orthorhomb. needles from Alc.
11-Desoxy-17-hydroxy- corticosterone	Plates	Ether	-----
17a-Dihydroequilin		Acetone	-----
Equilenin	Needles	dil. Alcohol	-----
Equilin	Orthorhomb. Sphenoidal plates	Ethyl Acetate	-----
Estradiol-17a	Prisms	80% Alcohol	-----

Table 6 Continued

Estrane-3a,17a, -diol	Benzene + Pet. Ether	Benzene + Pet. Ether	Methanol
5,6,8-Estratriene- 3b-ol-17-one	Needles MonoClin. Prism	Benezene- Ligroin dil. Methanol	Aq. Acetone
Estriol	Monoclin. crystals	dil. Alcohol	90% Alcohol
Estrone	Monoclin. + Orthorhomb.		Alcohol
Ethynyl Estradiol	Needles	Methanol + HOH	-----
Etiocholane	Needles	Acetone	-----
Hexahydroequilenin		Ethyl acetate	Platlets from Dil. alcohol
17a-Hydroxy-cortico- sterone	striated blocks	Abs. or Isopropyl alc.	Hygroscopic rods
Isoestradiol		dil. Methanol + Chloroform	-----
Isoestrone		Ether + MeOH	-----
17-Methyltestosterone		Hexane	-----
7-Oxoestrone		Alcohol	-----
Pregnane	Plates	Methanol	-----
Pregnan-3a,20a-diol	Plates	Acetone	-----
Pregnan-3a,20b-diol		Alcohol	-----
Pregnan-3b,20a-diol		Alcohol	-----
Pregnan-3b,20a-diol		Ethyl acetate + Pet. Ether	-----
Pregnan-3,20-dione	Needles	dil. Alcohol	-----
Pregnan-3a-ol-20-one		dil. Alcohol	-----

Table 6 Continued

Pregnan-20a-ol-3-one	Prisms	Acetone	-----
Pregnan-3b-ol-20-one		dil. Alcohol	-----
Pregnan-20b-ol-3-one		dil. Methanol	-----
Δ^4 -Pregnen-11b,17a, 20b,21-tetrol- 3-one	Hydrated crystals	dil. Acetone	-----
Δ^4 -Pregnen-17a,20b, 21-triol-3-one		Methanol	Acetone Ether
Pregnenolone	Needles	dil. Alcohol	Needles, Alcohol
Progesterone	Prisms + needles	dil. Alcohol	-----
Testosterone	Needles	dil. Acetone	-----

L. Dorfman, Chem. Revs., 53:47, 1953.

Sulphuric acid (conc.) spectra. -- This is the most routinely used method for the identification of steroids. This is carried out by placing 25 ug of the compound in 3.0 ml of concentrated reagent grade sulphuric acid and allowing the solution to stand for 2 hours at room temperature. Following the incubation period, the solution is read against sulphuric acid with the spectral analysis being carried out between 220 and 600 mu. Several hundred such spectra have been reported by Bernstein and Lenhard

S. Bernstein and R. H. Lenhard, J. Org. Chem., 18: 1146, 1953.

as well as others. By placing the spectral characteristics on punch key sort cards an unknown steroid spectrum can easily be solved by removal of the cards containing comparable spectral peaks. Occasionally two or more steroids will have the same spectral characteristics; however, usually the polarities and reagent tests are different enough to allow selection of the proper compound. Interpretation of spectra not recorded on the punch card system can be carried out by means of studies published by Bernstein and Lenhard as well as Linford.

S. Bernstein and R. H. Lenhard, J.O.C., 19:1269,1954;
J. H. Linford, Can. J. Biochem. Physiol. 35:299,1957.

Infrared Absorption. -- Characteristic group frequencies in the infrared have been reported by Jones and Herling for steroids. Also atlases of the infra-red

R. N. Jones and F. Herling, J.O.C. 19:1252,1954.

spectra of steroids have been published by Dobriner and Jones. This analysis can be carried out in a variety of solutions or in the solid form. This type of analysis is most useful during synthesis reactions as it is possible to follow the disappearance of a stretching band of a carbonyl group undergoing reduction with the concomitant appearance of a hydroxy frequency. It is equally useful in the elucidation of an unknown structure in a molecule.

Mixed Chromatography. -- Inasmuch as the polarity of the steroid determines the rate of mobility in a chromatography system, this differential polarity can be utilized to determine the structure and or difference in structure of a steroid. If two compounds are suspected of being identical in structure, they may be mixed and run in several different systems. Usually, if the two compounds are not resolved in systems of different solvent polarity, they can be considered as being identical. Occasionally

this criterion breaks down and the method should be viewed with caution if not coupled with other tests.

Melting points. -- All melting points were determined by the capillary-glycerin bath technique and are uncorrected.

Derivatives (Ester formation, Oxidation and Reduction).

Formation of Acetates. -- The formation of acetate derivatives allows the identification of different steroids that might possess the same polarity in the free alcohol form. Unhindered secondary alcohols form acetate derivatives at room temperature in pyridine and acetic anhydride. Hindered alcohols, such as the 11b-ol, require heating in the presence of an acid catalyst such as p-toluenesulfonic acid (p-TSA). Thus, it is possible to demonstrate by means of acetate derivative formation the presence of a hindered hydroxy group in the molecule in addition to the unhindered alcohol information.

Oxidation and reduction reactions of various steroid functional groups. -- Perhaps the most useful reactions for the identification of an unknown steroid are those of oxidative and reduction derivative formation. By these two methods it is possible to convert a suspected steroid structure to a known compound.

a. Oxidation with Sodium Bismuthate. To a 15 cc centrifuge

J. I. Appleby, Biochem. J. 60:453,1955; J. K. Norymberski, Nature 170:1074,1952; E. Diczfalusy, Acta.

Endo.27:275,1958.

tube, add about 50-100 micrograms of the steroid to be oxidized. If a quantitative Zimmerman is to be done, a blank tube must also be run in parallel. Add 4 cc of 50% acetic acid (v/v) to each tube. Place the tubes in a container which will shield all light from the reaction, permitting the top of the tubes to protrude for the addition of the oxidizing agent. Add to each tube about 50 mg of Sodium bismuthate, stopper, and shake. (The sodium bismuthate used must be checked periodically for oxidizing ability, by performing standard reactions with known amounts of cortisone.) Permit the tubes to shake on an automatic shaker for one hour at room temperature, keeping the reaction shielded from light at all times. At the end of an hour, add 10 cc of benzene, shake well, and centrifuge if necessary (usually not necessary). Remove the top benzene layer with a needle syringe, and re-extract twice more with benzene. Filter the combined benzene extracts through filter paper to remove any NaBiO_3 , washing the paper with benzene. Shake with 10 cc of saturated NaHCO_3 , washing the paper with benzene. Shake with 10 cc of saturated NaHCO_3 solution, to neutralize the acid, wash with a small amount of water, and dry over Na_2SO_4 . Evaporate the benzene, and transfer the residue to a small test tube for chromatography or chemical studies.

b. Oxidation with chromic acid. To 50 mgs of steroid to

S. Burstein, J. Biol. Chem., 213:581, 1955.

be oxidized in a flask, add 7.5 ml of 90% acetic acid containing 75 mgs of chromic acid (1 gram per cent). Shake at room temperature for 10 minutes. Add about 30 ml of MEOH, and evaporate to near dryness. Extract 3 x 1 vol with ETAC; after adding about 15 ml of HOH to the oily residue. Back-extract with HOH, and discard HOH. Dry ETAC solution over Na_2SO_4 , filter and evaporate to dryness. Transfer to small test tube for further study.

Procedure for periodate oxidation. Two methods were used: 1) The compound of residue is mixed with

C. DeCourcy, J. Biol. Chem., 229:935, 1957.

4 ml of 1% sulfuric acid in a centrifuge tube. 2 ml of sodium m-periodate is added and the tube kept in the dark for 2 hours at room temperature. The solution is neutralized with 0.5 ml of 10% NaOH. 10 ml of ether (or benzene) are added and the mixture shaken and then centrifuged. After this, the ether is removed. The extraction is performed twice more and the ether phases are combined and washed once with water. The ether is then dried and evaporated, yielding the oxidized residue.

2) Place the compound (or residue) to be oxidized in a ⁷⁷

J. Talbot and B. Eitington, J. Biol. Chem.,
154:605, 1944.

15 ml centrifuge tube and add 2.7 ml of absolute MEOH, 0.5 ml of a 50% aq-MEOH solution of 70 mgs. of HIO_4 , and 0.04 ml of conc-sulfuric acid. Allow to stand for one hour and transfer to a separatory funnel containing 180 ml of ether. Wash ether solution with 3 x 25 ml of 10% sodium hydrosulphite in 1 N solution of NaOH. Wash ether with 25 ml of 1 N NaOH solution and 4 x 25 ml of HOH. Evaporate to dryness.

c. Reduction with metal hydrides. The chemistry of metal hydrides is extremely complicated and a thorough knowledge of the chemosteriospecificity of the reactions should be available before such reductions are contemplated. Specific and identical reactions are morified by slight traces of alkali, increase or decrease in temperature and length of reaction time. The usual procedure calls for a four-fold Molar excess of the reducing reagent, which usually partition with the extraction solvent and are very troublesome when crystallizations are attempted. Some of the more commonly observed reactions are noted in Table 7.

Thus, when these reactions described above are

Table 7. Metal Hydride Reductions*

<u>Hydride</u>	<u>Substrate</u>	<u>Time</u>	<u>Temp.</u>	<u>Product</u>	<u>Yield</u>	<u>Other Prdts.</u>
KBH_4	20-one 11-one	O.N. [†]	Rm.	20b-OH 11-one	70%	20b-Oh 11b-OH
LiAlH_4	20-one 11-one	O.N., reflux [‡]	-10°C	20a-OH 11b-OH	52%	-----
LiAlH_4	20b-OH 11-one	O.N., reflux	-10°C	20b-OH 11b-OH	---	-----
LiBH_4	20-one 11-one	O.N., reflux	Rm.	20b-OH 11-one	63%	20b-OH 11b-OH
LiBH_4	20b-OH 11-one	O.N., reflux	Rm.	20b-OH 11b-OH	95%	-----

* All compounds had C-21-acetoxy structures

† With stirring overnight

‡ With stirring overnight, then refluxing for two hours

carried out, and tests prove the structure to be that of a⁷⁹ known compound, the structure of the parent compound has been elucidated. These methods permit the conversion of ketones to alcohols, thus permitting the preparation of new, additional acetate derivatives. Typical reaction utilized throughout this work are summarized in Table 8.

Elucidation of side chain structure following derivative formation

Following the isolation of the steroid by chromatography, elution and purification the side chain structure can be determined by the Axelrod method of micro-organic analysis. Further proof of the side chain structure can be obtained by derivative formation and analysis of the derivative. This permits additional chromatographic analysis as well as different spectral characteristics. Again, by this method, an unknown steroid can be converted to a known compound; if this converted compound, now tentatively a known compound, conforms to all the tests of an authentic sample of the suspected compound run simultaneously, the structure of the parent compound has been established. The usual reactions employed are presented in Chart V.

Preparatory reactions prior to derivative formation

Protective reactions, such as ketal formation by carbonyl groups, prior to reduction of selective site reactions are detailed in the synthesis section (See synthesis section). These reactions have been successfully carried out on quantities as small as 50 ugms. This type of manipulation affords

Table 8. Oxidation and Reduction Reactions of Steroid Functional Groups

Group	CrO ₃ -HAc	MnO ₂	NaBiO ₃	H ₅ IO ₆	NaBH ₄	KBH ₄	LiAlH ₄
3a OH	3-keto	---	3a OH	3a OH	---	---	---
3b OH	3-keto	---	3b OH	3b OH	---	---	---
4-3b OH	4-3-K*	4-3-K	4-3b OH	4-3b OH	---	---	---
6b OH	6-keto	6-keto, (reflux; 6b OH; shake)	6b OH	6b OH	---	---	---
11b OH	11-keto	11b OH	11b OH	11b OH	---	---	---
17b OH	17-keto	17b OH	11-keto 17-keto	17b OH	---	---	---
20a OH	20-keto	20a OH	20a OH	20a OH	---	---	---
20b OH	20-keto	20b OH	20b OH	20b OH	---	---	---
21-OH	---	21- OH	See Chart	---	---	---	---
3-keto	---	---	---	---	3b OH	---	3b OH(5a) 3a OH(5b)
4-3-keto	---	---	---	---	4-3b OH	---	20% a OH 80% b OH
11-keto	---	---	---	---	11b OH	11-keto	50% 11b OH
6- keto	---	---	---	---	6b OH	---	95% 6b OH 5% 6a OH
20-keto	---	---	---	---	20b OH	70% b OH	52% 20a OH
17-keto	---	---	---	---	17b OH	17b OH	17b OH

* Ketone

CHART V. STEROID SIDE CHAIN REACTIONS

References on Chart V

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1. F. Sondheimer, C. Amendolla and G. Rosenkranz, J.A.C.S., 75:5930, 1953.
 2. W. G. Dauben, R. A. Micheli and J. F. Eastham, J.A.C.S., 74:3852, 1952.
 3. E. P. Oliveto, C. Gerold and E. B. Hershberg, J.A.C.S., 76:6111, 1952.
 4. J. K. Norymberski, J. I. Appleby, G. Gibson and R. O. Stubbs, Biochem. J., 60:453, 1955.
 5. J. K. Norymberski and C. J. W. Brooks, Biochem. J., 55:371, 1953.
 6. R. Antonucci, S. Bernstein, M. Heller, R. Lenhard, R. Littel and J. Williams, J. Org. Chem., 18:70, 1953.
 7. J. K. Norymberski and G. F. Woods, J. Chem. Soc., 3426, 1955.
 8. L. F. Fieser and M. Fieser, Steroids, Reinhold Publishing Corp., New York, 1959, p.632-3.
 9. Ibid., p.567-8.
 10. W. Klyne, The Chemistry of the Steroids, Methuen Co., London, 1957, p.158.
 11. Ibid, p.80-3.
 12. S. Burstein and R. I. Dorfman, J. Biol. Chem., 213:581, 1955.
 13. Unpublished work, this laboratory.

14. N. Brooks and J. K. Norymberski, *Biochem. J.*, 55:371, 1953.
 15. E. Diczfalusy, L. O. Plantin and G. Berke, *Acta Endocrinol.*, 27:275, 1958.
 16. J. K. Norymberski, *Nature*, 170:1074, 1952.
 17. L. F. Fieser and M. Fieser, as above, p.268-70.
 18. Ibid., p.738.
 19. C. M. Southcott, H. E. Bandy, M. Darraah and S. E. Newcome, *Can. J. Physiol. and Biochem.*, 34:913, 1956.
 20. W. Klyne, as above, p.68.
 21. Ibid., p.93.
 22. Ibid., p.97.
 23. T. Reichstein, *Helt. Chim. Acta*, 20:978, 1937.
 24. I. E. Bush, *Biochem. J.*, 71:718, 1959.
 25. C. DeCourcy, *J. Biol. Chem.*, 229:935, 1957.
 26. No reaction site.
-

additional proof of structure of an unknown.

Synthesis

Fernholz and Stavely and others have noted that when

E. Fernholz and H. E. Stavely, Abstracts of the 102nd Meeting of the Am. Chem. Soc., Atlantic City, N. J., Sept. 8-12, 1941.

R. Antonucci, S. Bernstein, R. Littell, K. J. Sax, and J. H. Williams, J. Org. Chem., 17:1341, 1952.

P. L. Julian, E. W. Meyer and I. Ryden, J.A.C.S., 72: 367, 1950.

P. L. Julian, E. W. Meyer and I. Ryden, J.A.C.S., 71: 756, 1949.

P. L. Julian, R.P.H.R., 6:195, 1951.

a 4-3-ketosteroid is treated with ethylene glycol (in benzene with p-toluenesulfonic acid, p-TSA), the resulting ketal is formed with the coincident rearrangement of the double bond to the C 5,6 position; thus, during the reduction reactions the carbonyl was protected and could be regenerated by a variety of means. In this connection, Wendler demonstrated that the formation of a 21-acetoxy

N. L. Wendler, Huang-Minlon and M. Tishler, J.A.C.S., 73:3818, 1951.

derivative of a steroid hinders the condensation reaction of the ketal at the neighboring C-20 position. Also, the steric effects of the 21-acetoxy group to afford stereospecific reduction of the C-20 carbonyl group (also the C-11 position) to hydroxy-compounds in the beta configuration has been demonstrated by many workers such as Sarett.

L. H. Sarett, M. Feurer and K. Folkers, J.A.C.S., 73:1777,1951.

N. L. Wendler, as given above.

P. L. Julian, F. W. Meyer, W. J. Karpel and W. Cole, J.A.C.S., 73:1982,1951.

C. Djerassi, G. Rosenkranz, J. Pataki and S. Kaufmann, J. Biol. Chem., 194:115,1952.

G. Rosenkranz, J. Org. Chem., 17:290,1952.

That the hydrogen ion used in the synthesis comes directly from the reducing agent has been demonstrated by Kelmkelp

G. K. Kelmkelp, J. Org. Chem., 22:479,1957.

by using a labeled reducing agent (lithium aluminum deuterium).

No attempt has been made to establish conditions of time, temperature, pressure and concentration necessary to obtain the optimum yield of the compounds prepared. All

Melting Points (M.P.) are uncorrected, all Methanol (MEOH) spectra were run in spectrograde solvent and sulfuric acid spectra were in Reagent Grade. Spectra were either run on the Beckman DU Quartz or Cary Model 14-B spectrophotometers. All remaining solvents used were Reagent Grade.

All compounds synthesized in this study are summarized in Table 9.

Selective reduction following protective derivative formation

Preparation of 3,20 bisethylenedioxy- Δ^5 pregnane-17 α -ol (II)

Evaluation of the Procedure for Ketal formation. --

To evaluate the procedure for the preparation of ketals, Δ^4 pregnene-17 α -ol,3,20-dione (I) was used. This would form the 3,20-diketal, and the hydrolysis of the ketal could be studied by the reappearance of the Δ^4 -3-ketone and the return to the parent R_f in mixed chromatography, see Flowsheet 1. 123 mg of I was extracted and crystallized from a Merck saline suspension of I, control number 56R711. $\lambda_{\max} \text{H}_2\text{SO}_4$ 290, 440; $\lambda_{\min}$ 350. To a flask containing 120 mg of I, 7.5 mg of p-TSA in 10 ml of ethylene glycol was added and slowly distilled at 1.5 mm Hg and a still head temperature of 65 to 73° C. A magnetic plastic coated bar provided constant stirring. As the ethylene glycol was distilled off, 10 ml more was added containing 5.0 mg of p-TSA. The flask was removed after 3.5 hours and cooled. 70 ml of cold 0.1 N KOH was

Table 9. Summary of compounds Synthesized*

Chemical Name	Semi-Trivial	Usual abbreviation
1. Δ^4 pregnene-20b, 21-diol, 3-one	20b-OH-Deoxycorticosterone	20b-OH-DOC
2. Δ^4 pregnene-11b, 20b, 21-triol, 3-one	20b-OH-Corticosterone	20b-OH-CS
3. Δ^4 pregnene-17a, 20b, 21-triol, 3-one	20b-OH-Reichstein's S	20b-OH-S
4. Δ^4 pregnene-17a, 20b, 21-triol, 3, 11-dione	20b-OH-Cortisone	20b-OH-E
5. Δ^4 pregnene-11b, 17a, 20b, 21-tetrol, 3-one	20b-OH-Hydrocortisone	20b-OH-F
6. Pregnane-3a, 17a, 20b, 21-tetrol, 11-one	20b-OH-Tetrahydrocortisone	20b-OH-THE
7. Pregnane-3a, 11b, 17a, 20b, 21-pentol	20b-OH-Tetrahydrohydrocortisone	20b-OH-THF

* The use of a small "a" and "b" in chemical terms refers to Alpha and Beta configurations respectively.

Flowsheet 1. The addition and Removal of Ketals to 17 α -hydroxyprogesterone

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added and the flask allowed to stand at 5° C. for 4 hours. The flask was removed from the cold and extracted with 4 x 1 vol of ETAC. The organic phase was neutralized with saturated bicarbonate until bubbling no longer occurred. The ETAC was washed with HOH and dried over sodium sulfate followed by evaporation to a residue. Crystallization of the residue from acetone:MEOH afforded 115 mg of II. M.P. 196-197.5° C. (Yield 96%) $\lambda_{\max}$ None (MEOH); $\lambda_{\max}$ H₂SO₄ 300, 380, 445. $\lambda_{\min}$ 250, 350 (P), 390. See Spectrum No. I.

Regeneration of 3,20 carbonyl groups (I). -- Two different methods were used. 1) 8.0 mg of the diketal II was placed in a test tube containing 5.0 ml of acetic acid. This was placed in a boiling HOH bath for 90 minutes. After removal the contents were diluted to 50 ml with HOH and extracted with ETAC. The ETAC was neutralized, washed with HOH, dried and evaporated as described above. Crystalline material (UNweighed) appeared from chloroform:MEOH. $\lambda_{\max}$ 241 (MEOH); $\lambda_{\max}$ H₂SO₄ 290, 440. $\lambda_{\min}$ 350. Chromatography revealed one UV positive area which did not resolve from parent compound I. (100% yield) 2) 8.0 mg of the diketal II was placed in 20 ml of acetone containing 5.0 mg of p-TSA and was refluxed over night. Upon cooling, 10 ml of MEOH was added and the entire mixture was taken to dryness. Chromatography revealed two UV positive areas; one, of about 60%, with mobility of the parent compound I and another that was less polar. This had a mobility of 0.72 with respect to the 3,20-diketal I

and a mobility of 1.82 with respect to the parent compound I. This compound was not studied, however it was believed to be Δ^4 pregnene-17a-ol, 3,20-dione-20 ethylene ketal.

Preparation of Δ^4 pregnene-17a, 20b, 21-triol, 3, 11-dione (VI). See Flowsheet 2

21-acetoxy- Δ^4 pregnene-17a-ol, 3, 11, 20-trione (II), -- 1.5 mgs. of cortisone (I), (California Foundation, Lot No. 202416), was recrystallized and chromatographed in T/PG for 24 hours at 33° C. An UV absorbing area with the exact polarity of cortisone run on the side limb appeared at 14.0 to 17.6 cm. on 2.0 x 40.0 cm. paper. An approximate 25-ug area, more polar, was found as a contaminant and not identified.

About 100 mg of cortisone was converted by the addition of pyridine, acetic anhydride and being placed on the steam bath for 45 minutes to cortisone acetate. The resulting solution was eluted with HOH and the crystals collected by filtration and recrystallized from MEOH:HOH. The crystals were dried over night in a vacuum dessicator. M.P. 234-237° C.

21-acetoxy-3 ethylenedioxy- Δ^5 pregnene, 17a-ol, 11-20-dione (III). -- 80 mg. of II was refluxed for 4 hours with 2.5 ml ethylene glycol, 8.0 ml benzene, 2.5 ml toluene and 5.0 mg. p-TSA. The reaction heat was removed and the flask allowed to stand at room temperature for 16 hours. The flask was then transferred to -20° C. for 2 hours.

Flowsheet 2. The use of Ketals for carbonyl protection in selective reduction reactions

The crystals that formed were filtered and taken up in warm MEOH and recrystallized from MEOH:benzene. The needle-like yellowish crystals after drying had a M.P. of 265-268.5° C. and weighed 65 mg. (80% yield). λ_{max} None (MEOH), see Spectrum No. 2. Chromatography revealed no UV absorbing areas, yet a positive TPTZ was noted above the solvent front.

21-acetoxy-3 ethylenedioxy- Δ^5 pregnene-17a,20b-diol, 11-one (IV). -- The 3-monoketal III was dissolved in 10 ml of MEOH and 10 mg of sodium borohydride was added. The reaction was shaken in the cold for 3.5 hours. Cold HOH, with 10 drops of Acetic acid per 15 ml of HOH, was slowly added to the flask. A cloudy curd-like ppt appeared. The ppt was filtered and taken up in MEOH. After 3 days in the cold, crystals were obtained weighing 35.0 mg. (70% yield). Chromatography revealed a negative UV and TPTZ paper, except a very small amount of TPTZ positive (0.5%) substance on the starting line.

21-acetoxy- Δ^4 pregnene-17a, 20b-diol, 3, 11-dione (V). - The 3-monoketal was removed by placing 25 mg of IV in 15 ml of acetone and slowly adding 10 mg. of pTSA in 5 ml of MEOH. This was allowed to stand at room temperature for 24 hours and then refluxed overnight. 75 ml of HOH was added and the entire mixture extracted with ETAC, 1 x 1 vol, and chloroform, 3 x 1 vol. The organic phase was taken to dryness. 15 mg of white crystals was collected from ETAC: ether. M.P. 181.5-183° C. λ_{max} 238 (MEOH), $E = 15,300$;

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λ_{max} H_2SO_4 289, 345, 415. λ_{min} 245, 330, 385 (P). See Spectrum No. 3.

Δ^4 pregnene-17a, 20b, 21-triol, 3, 11-dione (VI). -- 25 ml of 10% KOH was heated to 45° C. overnight with 15 mg of V. The solution was neutralized and extracted with ETAC. The ETAC was evaporated to give a brown residue. The residue was chromatographed to give a positive UV and negative TPTZ area with the proper mobility of VI, R_F of 0.75 (60% yield): λ_{max} 238 (MEOH).

Preparation of Δ^4 pregnene-11b, 17a, 20b, 21-tetrol, 3-one

A procedure similar to that described above for the preparation of Δ^4 pregnene-17a, 20b, 21-triol, 3, 11-dione was carried out. After protective 3-monoketal derivative formation (63% yield) of the 21-acetoxy derivative of hydrocortisone, sodium borohydride reduction was carried out in the cold after column chromatographic separation of the reaction mixture. Hydrolysis of the ketal and regeneration of the Δ^4 -3-ketone was affected by hot acetic acid (68% yield). M.P. 210-218.5° C λ_{max} (MEOH) 242, $E = 15,600$; λ_{max} H_2SO_4 240, 283, 393, 467. λ_{min} 255, 355, 412. The spectra of the parent compound and the 20-b-hydroxy reduction product, both as the 21-acetoxy derivative, are presented in Spectrum No. 4.

Preparation of Δ^4 pregnene-20b, 21-diol, 3-one, Δ^4 pregnene-17a, 20b, 21-triol, 3-one and Δ^4 pregnene-11b, 20b, 21-triol, 3-one

These three 20 b-hydroxylated derivations were prepared in the same manner as just described.

Reduction of Tetrahydropregnene compounds

In as much as the tetrahydropregnenes are 3a-ol, no protective ketal derivative is necessary. Thus by selective reducing agents, it is possible to achieve C-20 carbonyl reduction without C-11 reduction in pregnane-3a, 17a, 21-triol, 11, 20-dione (I). This may be carried out with potassium or sodium borohydride in tetrahydrofuran and MECH respectively.

Preparation of 21-acetoxy-pregnane-3a, 17a, 20b, 21-tetrol, 11-one (III). See Flowsheet 3

100 mgs of I M.P. 226-229° C., is incubated at 37° C. for 12 hours with 100 mgs of potassium borohydride in 10 ml of tetrahydrofuran. After the incubation period 10 ml of water was added and 0.1 N acetic acid was added until bubbling no longer occurred. The reaction mixture is then extracted 3 x 1 vol with ETAC. The ETAC is washed with quarter vol of HOH, and the organic phase dried over dosium sulfate and evaporated. Crystals were obtained from MECH. M.P. 259-261° C. Chromatography revealed a TPTZ negative paper, but a positive area was noted to Axelrod's A to H reagents.

Preparation of 21-acetoxy-pregnane-3a, 11b, 17a, 20b, 21-pentol (IV)

6.0 mgs of II was available from U.S.P. Reference Standards, and was extracted from the talc with ETAC. The

Flowsheet 3. Selective reductions with different metal Hydrides

ETAC was taken to dryness and crystals appeared. To a ¹⁰⁰ methanolic solution of the crystals, 10 mgs of sodium borohydride was added and refluxed overnight. The sodium borohydride was destroyed by careful addition of 0.1 N acetic acid. The reaction mixture was extracted with ETAC, which was back washed with HOH, dried and evaporated. From MEOH fine needle like crystals appeared. Chromatography revealed one spot which was located with phosphomolybdic acid in ethanol. λ max H_2SO_4 273,300, 418,440 (I), 500, λ min 260, 283,360,490. See Spectrum No. 5.

Preparation of IV from I: Because of the unavailability of II, the preparation of IV in large quantity was attempted from I. To 100 mgs of I, 100 mgs of lithium aluminum hydride in 10 ml of disthyl ether was added and incubated at 37° C. for 48 hours. Cautious addition of HOH destroyed the reducing agent and the reaction mixture was diluted further and extracted with ETAC, which was then dried and evaporated. Fine crystals from MEOH: benzene were obtained. λ max H_2SO_4 285,312,355 (I),414, 450,502, λ min 256,280 (I),366. See Spectrum No. 6. In the chromatographic system of C/F, samples from A and B preparations did not separate.

Solvent Selectivity for Reduction Reactions

Because of the routinely poor yields of ketal derivatives, as well as concomitant loss of easily hydrolyzable (acid labile) functions elsewhere in the

SPECTRUM NO 5

SPECTRUM NO 6

molecule during reductions and extractions, a newly described procedure by Taub for the selective reduction

D. Taub, R. D. Hoffsommer and N. L. Wendler, J.A.C.S. 81:3291,1959.

of carbonyls was attempted. This procedure utilizes aqueous dimethylformamide as a solvent during mild reduction reactions, and the Δ^4 -3-ketone is less subject to attack. Also this procedure favors less acetyl migration between C-21 acetoxy and C-20 hydroxyl groups.

Experimental results, in this laboratory, are presented in Chart VI. From Chart VI, it is seen that following acetylation of (1) compound (2) was reduced with sodium borohydride in aqueous dimethylformamide. Extraction of the reactants and chromatography of the residue showed four areas by different testing methods already discussed. These results have been confirmed by a recent publication, in which Taub was able to isolate and

D. Taub, R. D. Hoffsommer and N. L. Wendler, J.A.C.S. 81:3291,1959.

identify one of the compounds resulting from such a reaction. This compound was shown to be the 20b-acetate-21-ol derivative (4) of their starting compound (2). However, they reported, in the absence of base catalysis

compound (4) was not formed. Evidently mild (1% base) ¹⁰⁴ catalysis is strikingly effective in promoting acetyl transfer from C-21 to C-20 in the above system. Unfortunately, a compound such as (4) would not permit the identification of the side chain by the usual micro-organic reactions employed through out this work. However, by chromic acid oxidation it is possible to demonstrate the structural difference between compound (3) and the acetyl migratory derivative (4). Compound (3) upon oxidation would form compound (7), while compound (4) would form the 20-acetoxy acid derivative (8). Thus, with the formation of compound (7), a positive alpha-ketol test would be obtained, while compound (8) would result in a negative. Indeed, the removal of the acetoxy group would result in the conversion of compounds (3), (4), (5) and (6) to compound (9), which is the desired 20b-hydroxy derivative of the starting material. Preparation of 21-acetoxy- ⁴ pregnene-11b, 17a, 20b, 21-tetrol, 3-one

To a stirred solution of 200 mgs of hydrocortisone 21-acetate in 8.0 ml of dimethylformamide at 20° C. was added 100 mgs of sodium borohydride in 2.0 ml of HOH. The starting material partly precipitated. Stirring was continued at 20° C. for four hours at which time all the material was in solution. Excess cold 10% acetic acid was added slowly, followed by HOH and the entire mixture was extracted with chloroform. The chloroform solution was washed with 2% potassium bicarbonate solution, water

Chart IV. Some unpredicted reduction reactions

and dried over sodium sulfate and evaporated. The residue was a brownish glass that resisted all attempts at crystallization. The glass was placed on neutral alumina column and elution affected with 50% benzene:chloroform to 100% chloroform. These two fractions were dried down and chromatographed on paper; it was first run in the T/PG system and then eluted and rerun in C/F system. A very large area with an R_F of 0.74 was found to be TPTZ negative, UV positive and to give a strong Axelrod reaction with reagent A to H and D to G. A small area (R_F of 0.11) was also found. This area was TPTZ positive and UV positive. This means it possessed an alpha, beta-unsaturated ketone as well as a dihydroxy-acetone side chain. Thus, it is obvious, this small amount of compound underwent some type of double bond rearrangement not anticipated by the reaction, It was not chemically identified.

Preparation of Δ^4 pregnene-17a,20b,21-triol, 3, 11-dione

Reduction of a suspension of 100 mgs of cortisone 21-acetate with 20 mgs of sodium borohydride in 5.0 ml of 80% aqueous dimethylformamide at 10° C. was carried out. The reaction was allowed to proceed for 5 hours. Excess 10% acetic acid, followed by water, was added. The solution was extracted with 3 x 1 vol of chloroform and the chloroform was back-extracted with 1 x 1 vol of 2% bicarbonate and then washed with HOH. The organic phase was

taken to dryness. Every 20 minutes during the reaction, an aliquot was removed for BTZ determination and also chromatographed. These data are presented in Charts VII and VIII respectively. It will be noticed in Chart VIII that chromatograms of fractions 1, 6 and 12 had areas more polar than the 20b-OH derivative. These were unidentified and are unexplained, however all but one are resolved in the final paper of fraction 18. Due to the nature of the polarity and negative UV and TPTZ, it is believed that this compound is 21-acetoxy-pregnene-3a, 17a, 20b, 21-tetrol, 11-one. The entire remainder of the sample was chromatographed after unsuccessful attempts at crystallization, and the 20b-hydroxy derivative eluted. Again crystallization was unsuccessful. A spectral analysis of the eluted compound checked with that of the 20-OH derivative prepared by the other method already described. See Spectrum No. 3.

Following 3 months storage at 20° C. the compound crystallized and was recrystallized to a M.P. 179.0 - 182.5° C.

CHART VIII

FRACTIONS

CHAPTER IV

RESULTS

Biological Data

Inasmuch as the development of hypertension followed approximately the same course once the surgery had been carried out, hypertensive dog 2-B was picked to exemplify the clinical changes noted in these animals.

Mongrel dog 2-B, female, with weight of 20.0 Kg prior to the initial surgery on 4/4/57, when the right kidney was tied with a "figure 8" ligature of umbilical tape as described in the Methods section. The post-operative history was uneventful. Twenty-six days later, on 4/30/57, dog 2-B weighed 18.9 Kg, at which time, the left kidney was tied in the same manner. Again, the post-operative course was uneventful. The dog was maintained on ad libitum mixed complete ration and water as prescribed, for any normal dog, by the Department of Physiology. Sixteen months later, on 7/15/58, the dog's blood pressure was determined to be 147/98 mm Hg. by an arterial puncture, using a pressure transducer connected to an amplifier in association with a direct writing apparatus that had been previously calibrated.

Nine months later, on 4/27/59, it was once again returned to the laboratory, and her blood pressure was determined to measure 260/140 mm Hg. At this time, the animal

weighed 19.2 Kg and appeared to be normal by physical observation. It was decided that the dog should be operated and the adrenal cannulated at this time.

The Adrenal Cannulation

Data pertaining to weight, sex, hours of collection and adrenal and heart weights of both the normotensive and hypertensive dogs are presented in Table 10.

It should be noted that the red cell volume remained relatively constant despite the collection of blood. In the normotensive group, Dog No. Normal, the hematocrit dropped from a value of 58% to 46% in spite of an approximate collection of 640 ml of blood via the cannulated adrenal vein. Likewise, the drop was approximately the same in the Hypertensive group as exemplified by Dog No. 5-S which changed from 59% to 49% during a period of 9 hours.collection.

In an attempt to determine whether or not a significant difference could be observed between the adrenal weight of the normotensive and hypertensive group, Table 11 was constructed. It can be seen that the average gram adrenal weight per kilogram body weight is 0.064 and 0.073 for the normotensive and hypertensive group respectively. All values were calculated for the right adrenal since the left was used for cannulation.

Following a short period of fluctuation, the adrenal blood flow remained relatively constant throughout the

Table 10. Biological Data obtained at time of Cannulation.

Dog No. Sex	Wt. Kg.	BP	Op.time hrs. mins	Collection Time Hrs. mins.		Organ Wts. gms.
E F	14.2	93/68	4 42	2	48	Ar ^a 1.229 1 1.279
6, M	12.2	-	4 10	4	36	Ar 0.583 1 0.568
3, M	19.0	-	4 40	3	17	Al 1.045 r 1.043
Normal, M	13.6	-	5 07	2	26	-----
2, M	12.2	-	3 30	4	37	Al 0.793 r 0.852
4, M	11.2	-	5 17	6	48	Al 0.543 r 0.677
HiT ^b 3-C, F	19.3	235/130	5 12	12	08	Al 1.513 r 1.606 Ht 115.51
HiT 5S, M	15.0	260/140	3 17	9	12	Al 1.513 r 1.354 Ht 137.99
HiT 2B, F	19.2	260/140	4 25	5	04	Al 1.143 r 1.276 Ht 145.98

Table 10. Biological Data obtained at time of Cannulation (continued)

Dog No Sex	Wt. Kg.	BP	Op.time Hrs. Mins.	Collection Time Hrs. mins.	Organ Wts. gms.
HiT 5(S1) M	13.6	232/160	2 42	11 10	Al 0.713 r 0.702 Ht 116.57

^a A means Adrenal, as Ht means Heart; r and l mean right and left

^b HiT stands for Hypertension

Table 11. Adrenal Weight vz Body Weight in Normotensive and Hypertensive Dogs.

Normal Dog No.	Ad.gms. (right)	Body Wt. (Kg)	Ratio gm/Kg	HiT ^a Dog No.	Ad.gms. (right)	Body Wt. (Kg)	Ratio gm/Kg
E	1.279	14.2	0.090	3-C	1.606	19.3	0.083
6	0.568	12.2	0.046	5-S	1.354	15.0	0.090
3	1.043	19.0	0.055	2B	1.276	19.2	0.066
2	0.852	12.2	0.070	5(S-1)	0.702	13.6	0.052
4	0.677	11.2	0.060				
Average			0.064				0.073 ^b

^aHit means Hypertensive group.

^bNo significant difference ($t=.40$ $p .50$)

remainder of the collection period, see Chart IX. This¹¹⁵ flow was stabilized by means of transfusion of either 50 or 100 ml of donor blood. A typical transfusion record and the maintenance of blood pressure is presented in Chart X. Therefore, it seemed of value to determine adrenal venous plasma flow per hour per kilogram body weight in the two groups; this data is set forth in Table 12. The calculations show the flow of plasma per hour per kilogram body weight to be 10.22 ml/Kg and 4.78 ml/Kg in the normotensive and hypertensive group respectively.

Because of the different units expressed in the literature, particularly that of Hechter, the flow value

O. Hechter, Amer. J. Physiol., 182:29,1955.

was also calculated in milliliters per hour per gram of adrenal per kilogram body weight. This type of expression appears in Table 13 for the normotensive group and in Table 14 for the hypertensive group.

This worker reports the range of flow values in 17 dogs to vary from 4 to 21 ml/hr/gram adrenal/Kg body weight. The average was 13.0 ml. This average value is well in keeping with our average of 13.08 ml in the normotensive group of dogs.

As stated above, prolonged surgical procedures may result in anoxia and thus reflect changes in the aldosterone

CHART IX

Table 12. Adrenal Plasma Flow Per Hour Per Kg. Body Weight.

Normal Dog No.	Wt. (Kg)	Time (min.)	Total ml flow	ml/hr Kg.	Hi T Dog No.	Wt. (Kg)	Time (min.)	Total ml flow	ml/hr Kg.
E	14.2	228	400	7.43	3-C	19.3	728	600	2.56
6	12.2	276	300	5.36	5-S	15.0	552	300	2.17
3	19.0	197	1000	16.25	2-B	19.2	304	700	7.17
Normal	13.6	146	3000	9.07	5(S-1)	13.6	610	1000	7.23
2	12.2	217	725	16.4					
4	11.2	408	800	10.82					
AVERAGE				10.89 ^a					4.78

^a Significantly larger (.02 p .05)

Table 13. Adrenal plasma flow in ml/hour/gm. Adrenal/Kg. body weight in Normotensive Dogs.

Dog No.	Wt. (Kg)	Flow ml/hr	Ad.Wt. (rt)	ML/hr/ gm.Ad.	ML/hr./gm.Ad./ Kg. wt.
E	14.2	105.3	1.270	82.3	5.80
6	12.2	65.5	0.568	115.3	9.45
3	19.0	304.2	1.043	291.7	15.35
2	12.2	200.0	0.852	234.7	19.24
4	11.2	117.8	0.677	174.0	15.54
Average				179.6	13.08 ^a

^a Not significantly different from hypertensive group found in Table 14, 0.30 p 0.50

Table 14. Adrenal plasma flow in ml/hour/gm. Adrenal/Kg. body weight in Hypertensive Dogs.

Dog No.	Wt. (Kg)	Flow ml/hr	Ad.WT. (rt)	ml/hr/ gm. Ad.	ml/hr/gm.Ad./ Kg. wt.
3-C	19.3	49.5	1.606	30.8	1.59
5-S	15.0	32.6	1.354	24.1	1.60
2B	19.2	137.7	1.276	107.9	5.62
5(S-1)	13.6	98.3	0.702	140.0	10.29
Average				75.7	4.78 ^a

^a Not significantly different from value reported for normotensive group, see Table 13.

production. This was not true in our experiments (See Table 15).

Gross Pathology (Hypertensive group)

Grossly the adrenal appeared normal, and the kidney was covered with fibrous connective tissue. The contralateral kidney, appeared normal in all respects. The heart appeared to have myocardial infarctions, both old and recent, as some areas were deep-red to brown, while others were a bright clear red.

Histopathology¹ (Hypertensive group)

There was some nodular cortical hyperplasia in the adrenal cortex. The zona glomerulosa was markedly increased in size. The only histopathology seen in the heart were anoxic changes in the myocardium of a very recent origin, perhaps within 0.5 hours before death.

The kidney that had undergone the trauma of the tie, demonstrated only slight ischemic changes plus the usual capsular fibrous changes. However, the contra-lateral kidney, showed thickening of the arterioles, protein precipitate in the tubules, with thickening of the glomerular tufts. Also there was occasional thickening and hyalinization of Bowman's membrane.

1. Doctor A. Horava of the Department of Pathology was kind enough to study the above sections.

Table 15. Steroid content at various times during the Cannulation procedure (reported as increase or decrease in values from starting level in tube one).

Dog No. tube & Hr.	BF area		Cortisol		Aldosterone		Cortisone	
	% inc	%dec	% inc	% dec	% inc	%dec	% inc	% dec
Normal (16, 8hr)	---	10.55	---	25.00	---	71.50	---	71.00
2B(HiT) (8, 1hr)	32.00	---	---	20.50	(unchanged)		---	27.20
(46, 4.5hr)	97.00	---	---	17.30	104.00	---	75.00 ^a	---
5S(HiT) (12, 5.5hr)	24.40	---	---	25.9	5.5	---	---	17.90
(19, 8.5hr)	82.4	---	---	20.30	65.30	---	(sample lost)	

^a This unexplained value may have been a laboratory error.

Chemical Data

The results obtained in this work are based on the following volumes of adrenal vein plasma collected at the time of cannulation of the adrenal vein:

DOGSNORMOTENSIVE

E - 400 ml
6 - 300 ml
3 - 1000 ml
N - 300 ml
2 - 750 ml
4 - 800 ml

HYPERTENSIVE

3-C - 600 ml
5-S - 300 ml
2-B - 700 ml
5(S 1) - 1000 ml

Therefore, the total volume of normotensive adrenal vein plasma is 3,525 ml, while the total hypertensive plasma used is 2,600 ml.

Following extraction, counter current distribution and chromatography of the individual samples of the normotensive and hypertensive adrenal vein plasma, the values of hydrocortisone, cortisone and aldosterone were quantitatively determined. The areas, BF, which were more polar than hydrocortisone, were pooled in the normotensive as well as in the hypertensive group in order to obtain sufficient material to permit the isolation and identification of the individual steroids present.

Following extraction, counter current distribution, and preliminary partitional analysis, the residue of the

extract was chromatographed in the systems already discussed in the Methods section. ¹²⁴

Chromatographic Data

Cortisol and less polar compounds

The initial chromatographic papers in T/PG from each normotensive and hypertensive adrenal vein blood extract were examined in UV light. Occasionally, representative strips were removed and either TPTZ or BTZ reactions carried out. This location of reducing steroids was done only when the UV absorbing area did not conform to our previous established criterion for the migration of expected areas. At this point, all areas of cortisol and less polar steroids were eluted from these initial papers and combined in test tubes; these test tubes contained all of the cortisol and less polar steroids extracted from each individual dog in both the normotensive and hypertensive series. In other words, all areas of cortisol and less polar steroids from each animal was combined and was separately rechromatographed in T/PG to remove more of the lipid material.

In the cT/EQ system, aldosterone will separate from cortisone and cortisol. After these secondary papers (T/PG) were removed and dried, they were again examined in the UV light. Two UV absorbing areas were seen; the more polar was cortisol and the less polar was the aldosterone-cortisone area. The aldosterone area, which lies equidistant between cortisol and cortisone, was not

present in amounts sufficient to be visualized in UV light.

Appropriate areas for cortisone, aldosterone and cortisol were eluted and taken to dryness in test tubes. The test tubes containing the aldosterone area from each paper was further chromatographed in the E_2B system in an attempt to remove any contaminants.

The test tubes containing the cortisol and cortisone areas were further purified by absorption on charcoal: celite (1:1) as described by Axelrod. Thus, semi-crystalline or flocculent residue was obtained in almost every tube.

Results of quantitative cortisol, aldosterone and cortisone analysis

These semi-crystalline residues, representing the cortisol and cortisone secretion of each dog, were diluted and reacted with the Porter-Silber reagent to quantitate the amount produced.

The appropriate areas on the E_2B papers were eluted and taken to dryness. The steroid content of each tube was determined by a quantitative BTZ reaction.

The results obtained, after correction for elutions, charcoal recovery and strip analysis, are presented in Table 16 as microgram/KG/Hr.

The BF areas or more polar than cortisol area

The BF areas from all papers of the normotensive group were combined in one test tube. The BF areas of the hyper-

Table 16. Concentration of cortisol, cortisone and aldosterone in adrenal plasma (all values given as microgram/Kg/Hr)

Normal Dog No.	Cortisol	Cortisone	Aldosterone	HiT Dog No.	Cortisol	Cortisone	Aldosterone
6	11.30	2.74	0.0685	5S	11.98	0.509	0.2730
4	8.02	3.59	0.0471	2B	22.49	1.870	0.1845
3	12.50	9.89	0.0337	5(S1)	14.93	2.980	0.0695
N	5.73	2.24	0.0660	3-C	16.66	0.790	0.0741
E	5.70	2.39	0.0406				
2	6.43	2.11	0.1390				
Average	8.28 ^a	3.83	0.0658		16.51	1.540	0.1503

^a Significantly different ($p < .01$)

tensive group were handled in a similar manner. This was ¹²⁷ done in order to achieve concentration sufficient to allow identification procedures to be carried out. These two combined areas were taken to dryness and stored at -20° C. in acetone. After 48 hours, a precipitate formed and was filtered in the cold and washed with acetone. These fractions were labeled as follows: N-BF (S) for the acetone soluble fraction of the BF areas from the normotensive group and N-BF (l_s) for the acetone insoluble material. The hypertensive group was given the same code except H- was used to designate it from the normotensive group.

All four fractions, N-BF(S), N-BF(l_s), H-BF(S) and H-BF(l_s), were chromatographed in C/F. They were allowed to run until cortisol would have moved approximately 35 cms. This exact movement was checked by running simultaneously on a side limb, lsatin, which has a mobility 0.95 of cortisol or tetrahydrocortisol in this system.

After chromatography, the papers were examined by means of UV light and Axelrod's Test VIII. Neither N-BF(l_s) nor H-BF(l_s) fractions contained any appreciable amount of steroids. The N-BF(S) fraction had no UV absorbing areas, while the H-BF(S) fraction showed two UV absorbing areas. These areas had R_F s of 0.67 and 0.78.

Preliminary micro-organic analysis

Two 0.3 cm strips were removed from each C/F paper for use in the analysis. On one, a TPTZ reaction was carried

out, revealing three areas containing dihydroxyacetone ¹²⁸ structures. These areas had R_F values of 0.27, 0.40 and 0.90. With the other strip an A to H reaction was carried out, which gave a positive area from 0.90 to 1.0. Both papers, N-BF(S) and H-BF(S), were eluted and rechromatographed in C/F, this time on narrower papers in order to concentrate the areas before performing further analysis.

The C/F run off fraction (RO), from H-BF(S) papers, was then taken to dryness and rechromatographed in T/PG. It was found to possess a UV absorbing area with R_F of 0.10. A strip, removed for a TPTZ reaction, gave negative results; however, the A to H reaction was positive for a C-20 reduction of the carbonyl in the dihydroxyacetone type group (glycol). This area was eluted and was added to the elution of the least polar compound on the C/F parent paper (H-BF(S)).

The second C/F paper was dried and examined by means of the Test VIII of Axelrod. This reagent demonstrated three positive areas for 3 alpha-ol type of compounds; however, since this paper did not represent resolved compounds, only those clearly separated could be recognized. This was followed by a PMA reaction, and four areas were found to be present with this reagent. (A) The two most polar areas, having R_F values of 0.27 and 0.35, were eluted. (B) The compound having an R_F of 0.70 was eluted. (C) The areas with R_F of 0.85 to 1.00 were eluted together.

Each part (A,B, and C) was re-chromatographed in 129
T/PG for seven days. A known amount of cortisol was added:
this permitted the reporting of R_F values, as the location
of cortisol is easily visualized in the UV and also reacts
promptly with TPTZ.

The results of each of the above three T/PG papers
will be discussed in turn.

Part A. -- UV examination of the dried paper revealed
one very polar area (R_F of 0.08). A TPTZ reaction was
carried out on a small strip, locating two areas, the pre-
viously mentioned UV area (R_F of 0.08), and an area having
an R_F of 0.55.

Part B. -- UV examination of the dried paper showed
one absorbing area with an R_F of 0.30. When and A to H
reaction was performed, a positive reaction was obtained
indicating the presence of a glycol side chain or a 20 re-
duced carbonyl. Following a TPTZ on another strip, two
positive areas were noted, R_F of 0.21 and 0.55.

Part C. -- No UV absorbing areas were located on the
dried paper. However, following a TPTZ reaction, one area,
having an R_F of 0.59 gave a positive result. On another
strip, a positive A to H reaction was obtained in the area
of R_F 0.05.

In an attempt to further resolve the areas on the a-
bove three chromatograms, each area located was eluted and
rechromatographed; each will be discussed in turn.

Elution of areas of Part A

The compound with an R_F of 0.08 was eluted and chromatographed in C/F paper, A-1, (with added cortisol) until the colored marker, Isatin, run on a side limb, reached the end of the paper. The compound with an R_F of 0.55 was eluted and combined with the 0.55 R_F of Part B, and run as paper B-2.

Elution of areas of Part B

The two compounds with an R_F of 0.21 and 0.30 were eluted together and rechromatographed in C/F, Paper B-1, (with added cortisol) until the colored marker reached the end of the side limb.

Elution of areas of Part C

The compounds with an R_F of 0.05 was eluted and rechromatographed in C/F, Paper C-1, in the same manner as described for Part A and B above. The compound with an R_F of 0.59 was eluted and divided into three parts. Fraction 1 was put through the Axelrod charcoal procedure and attempts at crystallization were carried out. A small amount of colloidal-like material appeared after three weeks in the -20° cold room. The test tube was centrifuged and the supernatant fluid decanted. The test tube was stored until a spectrum in sulphuric acid could be determined. On the second part, a NaBiO_3 oxidation was carried out. The resulting compound was stored until it could be combined

with the compound resulting from the more polar compound (R_F of 0.05) being chromatographed in the C/F system. Both of these were then rechromatographed in the B/F system.

Chromatography of areas from papers A, B and C

Paper A-1.- This C/F paper had one UV absorbing area with and R_F of 0.10. This area also gave a positive TPTZ reaction.

Paper B-1.- This C/F paper was examined by means of UV light, A to H and TPTZ. The following areas were observed:

<u>R_F values</u>	<u>UV areas</u>	<u>TPTZ areas</u>	<u>A to H areas</u>
0.13	neg	pos	neg
0.19	pos	neg	?
0.37	pos	neg	?
0.49	pos	neg	?
0.65	pos	neg	pos

Paper C-1.- This C/F paper contained one Test VIII (Axelrod) positive area, no UV absorbing areas and one area (corresponding to Test VIII area) that gave an A to H positive test having and R_F of 0.80.

Paper B-2.- This C/F paper contained two UV absorbing areas, R_F of 0.19 and 0.67. Neither of these two were TPTZ positive, but an area with an R_F of 0.36 was positive. An A to H positive test area was found with an R_F of 0.65.

Elution of Papers A-1, B-1, C-1 and B-2

Paper A-1.- The remaining area with an R_F of 0.10 was

eluted and stored.

Paper B-1.-- The entire area, more polar than 0.55, was eluted and will be combined with the corresponding area from Paper B-2; this sample was acetylated and run in C/F and called Paper B-1 plus B-2 (vP-Ac). The 0.65 area was eluted and will be combined with the 0.67 area of Paper B-2; this sample was run in T/PG and called Paper B-1 plus B-2 (0.65).

Paper B-2.-- The area 0.19 on this paper was eluted and combined with the polar areas on Paper B-1; the 0.36 area was eluted separately, as was the 0.67 area. The 0.36 area was run in T/PG and called B-3.

Paper C-1.-- The only area on this paper was eluted and stored.

Chromatography of eluted areas from Papers B-1, B-2 and C-1

Paper B-1 plus B-2 (0.65). - The eluted areas R_F of 0.65 and 0.67, of Papers B-1 and B-2 were chromatographed in T/PG (with added cortisol). On UV examination only one area was found with an R_F of 0.29. This area was also A to H positive.

Paper B-1 plus B-2 (vP-Ac).-- This C/F paper contained all of the areas more polar than 0.55 from both Papers B-1 and B-2. The eluted sample had been acetylated before re-chromatography had been carried out. Two areas were located; one with an R_F -Ac of 0.17 was UV negative and TPTZ positive, while another, with an R_F -Ac of 0.37 was UV

positive and A to H positive.

Paper B-3.-- This T/PG paper contained the 0.36 area from Paper B-2. No UV positive areas were located after chromatography, but two TPTZ positive areas were found. One had an R_F of 0.40, while the other was 0.59.

Chromatography of run-off from Paper RO-B-2.-- The run-off of Paper B-2 was chromatographed in T/PG (with cortisol added). Examination of the paper was negative for UV, but positive to TPTZ and positive to Test VIII for an area having an R_F of 0.60.

Elution of areas from Papers B-1 plus B-2 (0.65), B-1 plus B-2 (vP-Ac), B-3, RO-B-2, C and A-1 and tentative identification of areas

All areas in the above papers were eluted and placed in test tubes. In order to simplify coding, tentative identifications of the areas will be used hereafter in the following manner:

<u>PAPER</u>	<u>R_F</u>	<u>TENTATIVE IDENTIFICATION</u>
B-1 plus B-2(0.65)	0.29	20 beta-OH-cortisol
B-1 plus B-2(vP-Ac)	0.17	?
	0.37	?
B-3	0.60	THcortisone
C	0.59	20 beta-OH-THcortisone
RO-B-2	0.40	THcortisol
	0.59	allo-THcortisol
A-1	0.10	6 beta-OH-cortisol

All these areas were eluted and handled as described for area 0.59 of Paper C, except for the two areas from B-1 plus B-2 (vP-Ac). These two areas were only eluted and stored, as they were present in such small quantities that identification must await further collection of these two compounds. The remaining areas, as described, were divided into three parts:

- (1) for spectral analysis in either methanol or sulphuric acid or both,
- (2) for oxidative derivative formation,
- (3) unaltered sample for storage.

For clarity, the above data has been assembled in tabular form and is presented in Table 17.

Spectral analysis of Compounds

Following the methods of Axelrod for purification of eluted compounds (charcoal method) the samples were placed in the cold room ($-20^{\circ}\text{C}.$) for varying periods of time. Some of the samples appeared as a semi-crystalline flocculation and were centrifuged and the liquid discarded. Others did not appear in this state, were dried, and analysis was carried out on the residue. The results are presented in Table 18.

Oxidation Products

In an attempt to further elucidate the structure of the compounds that were isolated, it was necessary to prepare different oxidative derivatives of the parent compounds. Each oxidation was done to determine the maximal

Table 17. Summary of chromatographies and micro-organic analysis of isolated compounds.

Parent Compounds	Chromatography			Micro-organic analysis of side chain			
	T/PG, R _F	C/F, R _F	B/F, R _{THE}	17,20,21-triol	17,21-diol, 20-one	4-3-one	3a-ol
6 beta-ol-F	0.03	0.05	--	neg	pos	pos	neg
20 beta-ol-THE	0.05	0.80	0.20	pos	neg	neg	pos
THF	0.40	0.36	--	neg	pos	neg	pos
allo-THF	0.59	0.36	--	neg	pos	neg	pos
THE	0.60	1.00	1.00	neg	pos	neg	pos
20 beta-ol-F	0.29	0.67	--	pos	neg	pos	neg

Table 18. Spectral analysis of isolated compounds.

Compounds	λ ^{MEOH} max	λ ^{H₂SO₄} max	Molecular Ext. Coeff. ^c	Spectral values.
6 beta-ol-F	235.5	236	13,150 ^a	max. 240, 340, 400, 480, 550. min. 310, 380, 440, 530.
20 beta-ol-THE	none	414	13,550	See Spectrum No. 6
THF	none	330	12,050	See Spectrum No. 5
allo-THF	none	335	--- ^b	---
THE	none	335	14,260	See Spectrum No. 6
20 beta-ol-F	242	283	15,150	See Spectrum No. 4

^a Value taken from the literature

^b No standard value reported.

^c Determined in sulfuric acid

structures on the parent molecule. In Table 19, the 137
different types of oxidative derivatives that were prepared are presented. In Table 20, the chromatography of these oxidative derivatives plus the formation of normal and forced acetylation products are listed.

Quantitation of BF Compounds

By means of the usual formula for determining the molar concentration by means of the extinction coefficient, the values of each compound found in both the normotensive and hypertensive dogs blood are listed in Table 20.

Table 19. Type of Oxidation Carried out on Isolated Compounds.

Parent compounds	type of Oxidation	type of Resulting Compound
6 beta-ol-F	NaBiO ₃	6b,11b-diol,3,17-dione
	CrO ₃	3,6,11,17-tetraone
20 beta-ol-THE	HIO ₃	3a-ol,11,17-dione
THF	NaBiO ₃	3a,11b-diol,17-one
allo-THF	CrO ₃	3,11,17-trione
THE	NaBiO ₃	3a-ol,11,17-dione
20 beta-ol-F	HIO ₃	11b-ol,3,17-dione

Table 20. Chromatography of Oxidation of Products.

Resulting Type Compounds	T/PG	M/PG	Acetates Normal T/PG	Forced
6b,11b-diol,3,17-dione	R _T 0.25	-d-	R _a e0.95	-f-
3,6,11,17- trione trione	R _T 0.14	-d-	None	None
3a-ol,11,17-dione	R _T a2.70	R _T 2.34	M/PG R _E 2.70	None
3a,11b-diol,17-one	R _E b0.31	R _E 0.43	T/PG R _T 1.59	M/PG R _E 3.20
3,11,17-trione	R _E 0.92	-c-	None	None
3a-ol,11,17-dione	R _T 2.59	R _T 2.32	M/PG R _E 2.70	None
11b-ol,3,17-dione	R _T 0.92	R _T 0.14	None	T/PG R _T 1.65

- a Mobility is related to Testosterone
- b Mobility is related to 11-ketoetiocholanone
- c Only limited amount isolated.
- d System too slow to use
- e Mobility is related to 11 beta-ol-androstendione
- f Not done yet, see Discussion section.

Table 21. Quantitation of BF Compounds

Compounds	Normotensive		Hypertensive	
	Calculated ^a total conc	Quantity ^b /Kg/Hr	Calculated total conc	Quantity /Kg/Hr
6 beta-ol-F	---	---	4.48	2.930
20 beta-ol-THE	---	---	5.62	3.670
THF	2.97	5.298	4.75	3.105
allo-THF	---	---	1.44 ^c	0.943
THE	2.26	3.820	5.20	3.395
20 beta-ol-F	---	---	10 .01	6.600

^a Total value for adrenal plasma collected in Moles x 10⁻⁷.

^b Quantity secreted in Moles x 10⁻⁹/Kg body weight/hour.

^c No standard available; values reported as related to THF.

It would seem best to interpret the chemical data obtained from the plasma of the normotensive and hypertensive dogs before going into a discussion of the biological significance of these findings.

Interpretation of Chemical Data

As stated previously, two of the isolated compounds from the hypertensive dogs were only tentatively identified as no standard was available. Also, the synthesis of these two compounds is beyond the scope of this investigation. The four remaining compounds positively identified were synthesized in this laboratory. Samples of each compound isolated are being stored until such time as ultra-violet and infrared instrumentation becomes available on an ultra-micro scale.

The identification of each steroid unknown found in the adrenal venous plasma of the normotensive and hypertensive dogs will be considered in terms of its chromatographic, physical and spectral properties. Arrangement of these steroids is in descending order of polarity in the T/PG system of chromatography.

6 beta-hydroxy-cortisol

As can be seen from Table 17, this was the most polar of the compounds that have undergone analysis. This com-

pound did not reverse its polarity when chromatographed in the C/F system, thus giving a clue to the structure at C-11, i.e., the probable presence of a beta-hydroxy group. Due to the extreme polarity of this compound, with an R_F of 0.03 and 0.05 in T/PG and C/F respectively, / one-half of the compound was acetylated in order to facilitate the running rate of the compound. This was found to produce three spots in T/PG, with R_{F-Ac} of: 0.27, 0.92 and 2.37. At first this seemed very difficult to explain, however, subsequent analysis explained this observation.

As reported in Table 13, the compound was UV positive and gave a positive TPTZ reaction. Once this had been established, no other micro-organic reactions were carried out in order to conserve the small amount of compound present.

Oxidation of the remaining one-half of the free compound was carried out by means of two reagents. Oxidation with sodium bismuthate yielded a compound that had a R_T of 0.25 or 0.29 to that of 11 beta-ol-androstenedione. Since the quantity of this compound was extremely small, it was not run in any other systems, but eluted and saved for acetate studies.

The second oxidation was carried out by chromic acid and gave a product that had a mobility of 0.25 to that of 6 keto-androstendione (prepared from 6 beta-ol-Reichstein"S"),

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in the T/PG system. When this oxidation product was compared to testosterone, it ran with a mobility of 0.14.

Acetylation, under normal conditions, of the NaBiO_3 oxidation product, gave a single spot with a mobility of 0.95 to that of 11 beta-ol-androstenedione. Since this oxidation product was UV visible, the secondary, easily esterifiable, hydroxy group had to occupy some site on the oxidized molecule. Forced acetylation was not carried out, as the sample was saved for IR analysis at a later time.

Returning to the acetylated derivatives of the parent compound, it was decided to carry out a NaBiO_3 oxidation on all three compounds observed. Thus, this type of oxidation failed to alter the molecule that had an R_{F-Ac} of 0.25 in T/PG. This would indicate that the compound contained a C-21 acetate group, and thus prevented the oxidative removal of the side chain. Following elution, this compound was again normally acetylated; this resulted in a compound having a mobility of 2.35 times that of F-Ac, or equal to the third spot noticed following the first acetylation.

Bismuthate oxidation of the spot with an R_{F-Ac} of 0.92, resulted in the formation of a new compound. This new compound had a mobility of 0.93 to that of the acetate derivative of the bismuthate oxidation of the parent compound. On mixed chromatography the two compounds did not separate.

Therefore, it follows, that the possible identity of

the three spots from the acetylation of the parent com-¹⁴⁴
pound were: 1) the most polar, 0.27, was the C-21 acetoxy
derivative of 6 beta-ol-F, 2) the spot with a mobility of
0.92 was the 6 acetoxy derivative of the parent compound,
and 3) the compound with the 2.35 times F-Ac was the
6,21-diacetoxy derivative of 6 beta-ol-F.

Spectral analysis was carried out on the compounds
throughout the study. The parent compound had a spectrum
as reported in Table 18. This compares closely to that
reported in the literature. The MEOH spectrum had a peak
at 235.5 mu, which is characteristic of compounds con-
taining both a Δ^4 -3-ketone and a 6 beta-ol complex. The
unacetylated, bismuthate oxidation product also gave this
type of peak in MEOH.

Thus the tentative identification of this compound
as ⁴-pregnene-6-beta,11 beta,17 alpha,21-tetraol,3,20-
dione has been presented. Final identification must a-
wait IR analysis of the stored compound and derivatives.

20 beta-ol-tetrahydrocortisone

As can be seen in Table 17, the mobility of this
unknown was reversed, from a R_F of 0.05 in the T/PG
system, to a R_F of 0.80 in the C/F system. This is very
reminiscent of the behavior of THE, which runs with an
 R_F of 1.0 in C/F and in the T/PG system it has a R_F of
only 0.60. Thus, it would seem understandable that the
C-20 dihydro-derivative of THE, with only the change of

two hydrogen groups, should behave in a similar manner in¹⁴⁵ the same system. Then, also, its subsequent mobility in the B/F system, which is a less polar system, again places the compound at an R_{THE} of 0.20 which one would expect. Thus through these three systems, the mobility of the unknown was matched with the standard that had been synthesized for this purpose. As far as the micro-organic analysis, according to the procedure of Axelrod, the side chain was negative for the dihydroxy-acetone structure, positive for the glycol structure and gave positive results for a reduced A ring; this positive result is in agreement with the non-absorption of UV light.

When oxidation was carried out with HIO_3 , the product behaved as the 3 α -ol,11,17-dione derivative since it had a mobility of 2.70 and 2.34 of testosterone in the T/PG and M/PG systems respectively. Additional proof of the 3 α -ol structure can be seen by the easily formed acetate under normal conditions, as a 3 β -ol compound requires a catalyst (forced acetylation). To demonstrate that no hindered hydroxy groups existed on the molecule, a forced acetylation was carried out. The compound resulting from this reaction failed to separate from a sample of the starting compound. At no time did this oxidation product resolve from an authentic sample of 11-keto-etiocholanone, prepared from tetrahydrocortisone, when run on companion chromatograms. Both the unknown

oxidative and prepared oxidative compound failed to absorb UV light and were located by means of the Zimmerman reagent, ¹⁴⁶

Following acetylation of the parent unknown, 20 beta-ol-THE, the spectrum was determined in sulfuric acid. The spectrum was found to be consistent with that of the synthesized compound, 20 beta-ol-THE-diacetate, which is shown in Spectrum No. 6, in the synthesis section.

Thus the identification of 20 beta-ol-THE as pregnane-3 alpha,17 alpha,20 beta,21-tetraol,11-one has been established.

Tetrahydrocortisol

The identification of this unknown was carried out in much the same manner as described above. The mobility of this compound in two different systems, T/PG and C/F, checked with that of an authentic standard supplied by the Steroid Division of the U.S.P. and was found to possess a dihydroxy-acetone type side chain by means of TPTZ. It was also UV negative.

Oxidation of the unknown was carried out by means of NaBiO_3 , which would result in a 3 alpha,11b-diol,17-one type of compound. Chromatographic behavior in the T/PG and M/PG systems was consistent with that of 11-beta hydroxy-etiocholanone, as the mobility was 0.31 and 0.43 that of 11-keto-etiocholanone in the respective systems.

Additional proof of the structure of the oxidation

product resulted from the normal and forced acetylation¹⁴⁷ reactions. The normal acetylation reaction yielded a compound that had a mobility of 1.59 to that of testosterone in the T/PG system. However, upon doing a forced reaction, the resulting compound had a mobility of 3.20 of 11-ketoetiocholanone. This is proof of acetylation of some hindered hydroxy group, such as the 11 beta hydroxy group found in THF. It should be stated that this reaction could take place at any hindered hydroxyl, however, a hindered hydroxy group other than at the 11 position is unlikely due to the polarity of the parent oxidation compound, as its properties were consistent with an authentic sample carried through out the entire procedure.

The sulfuric acid chromogen spectral study of the parent compound was consistent in all respects with that of an authentic standard supplied by the U.S.P.

Thus by chromatographic, physical and spectral procedures, the identity of THF as pregnane-3 alpha,11 beta, 17 alpha, 21-tetraol, 20-one has been established.

Tetrahydrocortisone

This compound exhibits chromatographic properties that are characteristic of tetrahydrocortisone. That is, it reverses its polarity with respect to cortisol (in T/PG) when run in the C/F system as is shown in Table 17. Thus in T/PG it has a polarity of 0.60 R_F , while in C/F it will not separate from cortisol. This compound

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was UV negative which is indicative of the reduced ring A
and TPTZ positive for the dihydroxy-acetone side chain.

Further proof of its structure was obtained by preparing the oxidation product with NaBiO_3 , which seemingly resulted in a 3 α -ol,11,17-dione type of compound. This was established by chromatography in the T/PG and M/PG systems, in which the mobility to testosterone was 2.59 and 2.32 respectively.

Proof of the 3 α -ol structure was obtained by means of locating a UV negative and Zimmerman positive spot, with a mobility of 2.70 to 11-ketoetiocholanone in M/PO, after normal acetylation. The resulting compound of a forced acetylation, ran with the compound resulting from the normal acetylation. This is proof of an 11-keto group as it did not resolve from an authentic standard of 11-ketoetiocholanone carried through the entire procedure.

The sulfuric acid spectrum of the parent compound was similar in all respects to that of authentic THE.

Therefore, from the above data, the compound THE has been established as pregnane-3 α ,17 α ,21-triol, 11,20-dione.

20-beta-hydroxy-cortisol

Since this compound was found in the largest quantity, the following additional procedures were carried out upon it. This is another compound, whose chromatographic properties tend to establish its identity. In the T/PG

system it has an R_F of 0.29, while in C/F it has a mobility¹⁴⁹ of 0.67 to cortisol. This compound does not contain a reduced ring A. and thus is visible in UV light. Once the side chain was positively established as not reacting to TPTZ, no strips were cut to locate it on subsequent chromatograms. Micro-gram quantities of 20 α -ol-F were available as a side product in the synthesis work of preparing the 20 β epimer standard. The micro-gram quantity of the 20 α epimer was mixed with the unknown and chromatographed in T/PG and then again in C/F. In both systems the two epimers separated, thus establishing the unknown as the 20 β epimer.

Oxidation was carried out using HIO_3 and the resulting compound was the 11 β -ol,3,17-dione. This was proven by its mobility in T/PG of 0.92 to testosterone and 0.14 of testosterone in C/F. When this derivative was run as a mixed chromatogram with authentic oxidized 20 β -ol-F, the sample did not resolve in the two above systems.

Upon normal acetylation, the resulting compound did not separate from the starting oxidized sample, thus establishing the absence of any easily esterifiable secondary alcohol group. However, upon forced acetylation, a new product was obtained that had a mobility of 1.65 to that of testosterone thus establishing the presence of a hindered hydroxy group on the molecule.

Further proof of this hindered hydroxy group was ob-

tained by taking a small amount of the oxidized parent compound and reacting it with CrO_3 . The compound from this reaction was chromatographed in M/PG and it did not separate from an authentic sample of 11-ketoandrostenedione. This established the existence of the hindered hydroxy group at carbon-11. After forced acetylation of this CrO_3 oxidation product, it failed to separate from 11-ketoandrostenedione.

Elution of the mixed chromatogram of the 11 β -ol,3,17-dione oxidation product and the authentic sample of 11 β -hydroxy-androstenedione was carried out. This mixed sample was compared with the authentic sample by means of a sulfuric acid spectrum. The spectra were identical. Likewise, this was true for the CrO_3 oxidation product and 11-ketoandrostenedione. The spectrum of the unoxidized parent compound was similar to that of 20 β -ol-F.

Therefore, the identity of 20 β -ol-F has been established as ⁴-pregnene-11 β ,17 α ,20 β ,21-tetraol,3-one.

Allo-Tetrahydrocortisol

The identity of this compound can not be established in this laboratory due to the lack of a standard sample. The synthesis of this compound would be a major undertaking, as it must be prepared from some precursor possessing a 5 α -hydrogen configuration. Therefore, on the grounds of its mobility in T/PG and C/F, coupled with the oxidative product data, it is tentatively called allo-THF. Infrared

CHAPTER V

DISCUSSION

Studies relating to C-6 beta hydroxylation and C-20 alpha and beta reductions in bio- logical systems

In the past several years, the increased frequency of reports concerning atypical hydroxylations have become numerous. Previously it has been thought that hydroxylation of the carbonyls meant a reduction in the biological potency of a compound and also an increased tendency to be excreted, via the kidney, as the hydroxyl then constituted a site for conjugation. This reasoning has remained unshaken for the carbonyl of the Δ^4 -3-ketone of ring A; however, hydroxylation of such sites as C-6 and reduction of C-20 carbonyls have not been proven to enter into this type of biological inactivation.

Furthermore, Zander and co-workers have demonstrated

J. Zander, J. Clin. Endo. Met., 18:337, 1958.

that carbonyl reductions at C-20 of progesterone to the 20 alpha (20a) and 20 beta (20b) epimeric forms, results in the former compound being twice as biologically active and the latter 2/5 as active as progesterone when assayed by the Hooker-Forbes method. This was, perhaps, the first demonstration that these atypically hydroxylated deriva-

tives may alter the biological activity in either direc-152
tion from that of the parent compound.

In the light of the results from this investigation,
it seemed in order to review the studies related to C-6
hydroxylation and C-20 reductions.

Abelson was able to demonstrate the isolation of

D. Abelson, F. Ulrich and C. N. H. Long, Proc. Soc.
Exptl. Biol. Med. 89:386, 1955.

the 20 beta epimer of 20-OH-cortisol from rat plasma after
the administration of the parent compound, cortisol. 20
alpha dihydrocorticosterone was identified by Southcott
in the blood of normal men after large infusion of corti-
costerone.

C. M. Southcott, Can. J. Biochem. Physiol. 36:819,
1958.

In an exhaustive study Neher and Wettstein were able

R. Neher and A. Wettstein, Helv. Chim. Acta 39:2062,
1956.

to isolate nine pregnane derivatives from adrenal tissue
incubations. Five of these compounds were new and none of

the others had previously been obtained from adrenal 153
tissue. Three of these nine compounds were 6 beta-ol pro-
ducts, one a 20 alpha and one a 19-ol derivative. These
were isolated from either swine or bovine adrenals and were
fully characterized. A possible intermediate between cholest-
erol and pregnenolone has been reported by Solomon. When

S. Solomon, P. Levitan and S. Lieberman, Proc. Can.
Physiol. Soc., 20th Meeting, 54, (Montreal, Canada, 1956).

beef adrenal homogenates were incubated with cholesterol-
 C^{14} , 20 beta-OH-cholesterol- C^{14} was formed, but none of the
following cholesterol derivatives were radioactive: 22-alpha-
OH, 22 beta-OH or 22 keto.

Lombardo and coworkers succeeded in demonstrating the

M. E. Lombardo, J. E. Roitman and P. M. Hudson, J. Clin.
Endo. Met. 16:1283, 1956.

presence of 6 beta hydroxylase activity in human adrenal
gland slices, when progesterone- C^{14} was converted to 6 beta-
OH-cortisol- C^{14} by means of their incubation procedures.
unfortunately, identification procedures for 20 reduction
products were not carried out.

In the washed residue (5000 x G) of an adrenal bovine
homogenate, Meyer demonstrated the conversion of androstene-

dione to the corresponding 6 beta-OH- derivative.

A. S. Meyer, M. Havand, M. C. Lindberg, M. Gut and O. G. Rogers, *Acta Endocrinol.* 18:148,1955.

Burstein has demonstrated the presence of a 6 beta-ol

S. Burstein, R. I. Dorfman and E. Nadel, *Arch. Biochem. Biophys.* 53:307,1954.

S. Burstein and R. I. Dorfman, *J. Biol. Chem.* 213:581, 1955.

E. M. Nadel, S. Burstein and R. I. Dorfman, *Arch. Biochem. Biophys.* 61:144,1956.

products in the urine of guinea pigs as well as the urine of normal ACTH stimulated men. Cortisol administered to guinea pigs yielded 6 beta-OH-cortisol along with the two 20 epimeric reduction compounds of cortisol.

Dog liver perfused with testosterone afforded the identification of 6 beta-OH products of testosterone and its 17 oxidation product, androstenedione, in the hands of Axelrod.

L. R. Axelrod, L. L. Miller and F. Herling, *J. Biol. Chem.* 219:455,1956.

Following this work further, Forchielli and Dorfman¹⁵⁵ found the microsomes of rat liver to possess the 6 beta

E. Forchielli and R. I. Dorfman, J. Bio. Chem. 223:443, 1956.

hydroxylase activity and they found that 11-desoxy-cortisol was converted in high yield to 6 beta-OH-11-desoxy-cortisol by their preparation from rat livers.

Human placenta perfusions with progesterone, carried out by Berliner, demonstrated the presence of 6 beta-OH-

D. L. Berliner and H. A. Salhanick, J. Clin. Endo. Met. 16:903, 1956.

progesterone, Hayano under slightly different experimental

M. Hayano and R. I. Dorfman, J. Biol. Chem. 211:227, 1954.

conditions, had isolated the 6 keto derivativ of progesterone. She has also been able to demonstrate the presence of the 6 beta-OH- activity in bovine corpus luteum homogenates, and was able to isolate the 6 hydroxy product of desoxycorticosterone when this tissue was used.

M. Hayano, M. C. Lindberg, M. Wiener, H. Rosenkratz
and R. I. Dorfman, *Endocrinol.* 55:326, 1954.

Caspi and Hector have perfused cortisone through rat

E. Caspi and O. Hechter, *Arch. Biochem. Biophys.* 61:
299, 1956.

livers. They have been able to isolate and identify ten
metabolites, four of which were also reported by Axelrod and
Miller. Of particular interest in the work of Caspi is the

L. R. Axelrod and L. L. Miller, *Arch. Biochem. Biophys.*
49:248, 1954.

finding of four metabolites reduced in the 20 beta- form,
and the absence of the 20 alpha- epimer.

Of marked interest is the finding that when similar
experiments are carried out by perfusing livers, which have
been experimentally made cirrhotic, there results the
formation of an additional hydroxylation of the perfused
product, namely 6 beta-OH cortisone. An unexplained, and
unexpected hydroxylatin found by Axelrod and Miller was
that of the formation of a 2 beta-OH compound.

L. L. Miller and L. R. Axelrod, Metabolism, Clin. and Exptl., 3:438,1954.

Later, identical studies with cortisol did yield only five metabolites as expected; two 17-ketosteroids and three other compounds of which one was found to be a 20-beta-allo-pregnane pentol. This work was by Caspi and Hechter.

E. Caspi and O. Hechter, Arch. Biochem. Biophys. 52: 478,1954.

6 beta hydroxylation also occurs in the estrogen series. Only one such study will be cited. Mueller and Rumney found mouse liver microsomes, in the presence of

G. C. Mueller and G. Rumney, J.A.C.S. 79:1004,1957.

TPNH and oxygen, hydroxylated estradiol-16-C-14 to produce 6 beta-OH-estradiol and 6-Keto-estrone.

Hubener has conducted extensive studies on the re-

H. J. Hubener, R.P.H.R. 12:146,1956.

H. J. Hubener, D. K. Fukushima, and T. F. Gallagher, J. Biol. Chem. 220:499,1956.

H. J. Hubener, and J. Schmidt-Thome, Hoppe-Seyler's

duction at C-20 in the corticoid series and believes that a factor of substrate specificity is involved. They have concluded that the 20 beta epimer is formed predominately when the substrate has an 11 beta-OH group, and that the 20 alpha epimer is the chief reduction product when a carbonyl or desoxy- group is present at C-11. However, a close study of the above cited reports fail to support their conclusions.

Ulrich reports the principal metabolites of cortisol

F. Ulrich, Biochem. J. 68:361,1958.

by rat liver in vivo are four 20 beta reduction products, namely, 20 beta-OH-THcortisol, 20 beta-OH-allo-THcortisol, 20 beta-OH-cortisol and 20 beta-OH-allo-THcortisone.

Studies by Recknagel have shown the C-20 keto reductase

R. O. Recknagel, J. Biol. Chem. 227:273,1957.

of rat liver to be associated with microsomes and that a TPNH generating system is required for its activity.

In vitro metabolism studies with 11-desoxy-cortisol are reported by Forchielli and Dorfman. These were carried out by incubations with rat liver homogenates and twelve

E. Forchielli and R. I. Dorfman, J. Biol. Chem. 223: 443, 1956.

metabolic end products were identified. These isolated substances are important in that they demonstrated the reduction of ring A to both 5 alpha (allopregnane or androstane) derivatives and 5 beta (pregnane or etiocholane) derivatives. Further, they found two 20 beta reduced and one 20 alpha reduced metabolites in addition to one 6 beta-ol pregnene. From their data they reason that for each Δ^4 -3-ketosteroid, two Δ^4 hydrogenases are present in rat liver; one, which orients the reduction of the Δ^4 group to the 5 alpha configuration, while the second forms the 5 beta configuration.

As shown, Forchielli and Dorfman have found both 20 alpha and 20 beta reduction to occur with 11-desoxycortisol. Further, DeCourcy and Schneider have incubated

C. DeCourcy and J. J. Schneider, J. Biol. Chem. 223: 865, 1956.

C. DeCourcy and J. J. Schneider, Chemistry and Industry 315; 1956.

two steroids, both devoid of an 11 beta-OH group and found 20 beta reduction products; also, 17 alpha-OH-progesterone

yielded both the 20 alpha- and 20 beta derivatives.

Caspi has succeeded in preparing a specific enzymatic

E. Caspi, M. C. Lindberg, M. Hayano, J. L. Cohen, M. Matsuba, H. Rosenkratz and R.I. Dorfman, Arch. Biochem. Biophys. 61:267,1956.

reductase, that is, the 20 alpha orienting enzyme from hog liver. Bovine kidney mince when incubated with cortisol resulted in the formation of $C_{19}O_2$ and $C_{19}O_3$ steroids, as well as cortisone and 20 beta-OH-cortisol, indicating that this tissue contains a side chain splitting enzyme, and 11 beta-dehydrogenase, and a 20 beta hydroxylase. Ganis was not able to demonstrate any of the 20 alpha epimeric forms.

F. M. Ganis, L. R. Axelrod and L. L. Miller, J. Biol. Chem. 218:841,1956.

Later, DeCourcy was able to show that the homogenate

C. DeCourcy, J. Biol. Chem. 229:935,1957.

of rat kidneys in the presence of a TPNH generating system reduced the 20 carbonyl group to the 20 beta-OH group much the same as intact kidney cells.

As is usual, one finds isolated reports that do not

agree with the bulk of the literature. This is true when considering the 20 beta-hydroxylase as the selective reducing enzyme in liver tissue. When Caspi investigated

E. Caspi, Arch. Biochem. Biophys. 61:267,1956.

porcine liver brei, he found 20 alpha reductions taking place. In this study he utilized 11-desoxycortisol and all the metabolic products were alpha oriented in structure. Likewise, DOC resulted in a 20 alpha oriented metabolites. Taylor also reports that rabbit liver homogenates convert

W. Taylor, Biochem. J. 60:380,1955.

progesterone to the 20 alpha-ol pregnane derivatives.

Glick was able to isolate the 20 reduction products

J. J. Glick, Endocrinol. 60:368,1957.

of both the alpha and beta forms, of cortisone, THcortisone and THcortisol from the bile of cattle. He obtained these in crystalline form and proposed this route as a mechanism for the elimination of steroids in this animal; thus, accounting for the abnormally low urinary values of the compounds.

The metabolism of cortisol incubated with mouse

connective tissue was studied by Berliner and Daugherty. 162

D. L. Berliner and T. F. Daugherty, Proc. Soc. Exptl. Biol. Med. 98:3,1958.

They found, among other unexpected metabolites, one that was reduced at the C-20, but were not able to identify the orientation.

In a heart-lung preparation studied by Travis and Sayers,

M. Travis and G. Sayers, Endocrinol. 62:816,1958.

it was found that when C¹⁴-cortisol was added to the perfusate, ten percent of the radioactivity could be recovered from the homogenized tissue and blood in the form of 20 beta-OH-cortisol.

In an experiment of similar design, DeVenuto and Westphal isolated 20 beta-OH-cortisol from the muscles of the rat five minutes after the intracardiac injection of C¹⁴-cortisol. These workers did not study the blood of their animals.

F. DeVenuto and U. Westphal, Fed. Proc. 17:211,1958.

Thus we see from the literature a predominance of beta oriented reduction of the C-20 carbonyl. However, the

major urinary metabolic end products are found to be 20 alpha oriented as will be seen in the following literature.

As long ago as 1953, Lieberman was able to demonstrate that normal urine contained several allopregnanes that were 20 alpha reduction products.

S. Lieberman, J. Biol. Chem. 204:491, 1953.

This experiment was closely followed by one carried out by Engle. He studied the metabolism in vivo of

L. L. Engle, P. Carter and L. L. Fielding, J. Biol. Chem. 213:99, 1955.

corticosterone in a human. Of the four steroids isolated from the urine, there was an unusually increased concentration of 5 alpha (allo) derivatives; one was of the 5 beta configuration, which was also reduced at the C-20 in the alpha form.

In the guinea pig, Peron and Dorfman, found that the

F. G. Peron and R. I. Dorfman, Endocrinol. 62:1, 1958.

administration of ACTH resulted in a two to eight fold increase in the amount of cortisol, cortisone, 6 beta-OH-

cortisol and 2 alpha-OH-cortisol, besides the epimeric 20-OH reduction products of cortisol. However, the alpha form represented the greater per cent of reduced compounds.

Peterson reports the isolation of the 20 alpha

R. E. Peterson, C. E. Pierce and B. Kliman, Arch. Biochem. Biophys. 70:614;1957.

epimer of administrated cortisol in the human. Likewise, Bongiovanni and Eberlein have reported the 20 alpha epimer

A. M. Bongiovanni and W. R. Eberlein, Pediatrics 16: 628,1955.

in the urine of a patient having hypertensive congenital adrenal hyperplasia. In a later study these metabolites were isolated in crystalline form.

W. R. Eberlein and A. M. Bongiovanni, J. Biol. Chem. 223:85,1956.

Fukushima and Gallagher, in another study of the urinary excretion products of a patient with adrenal hyperplasia,

D. K. Fukushima and T. F. Gallagher, J. Biol. Chem. 226:725,1957.

D. K. Fukushima and T. F. Gallagher, J. Biol. Chem. 165
229:85,1957.

D. K. Fukushima and T. F. Gallagher, J. Clin. Endo.
and Met. 18:694,1958.

report the isolation of four 20 alpha epimers in the urine.
These were of the pregnane and allopregnane series.

These findings have further been substantiated by the
work of Rosselet et al. who found the principal pregnane

J. P. Rosselet, J. W. Jailer and S. Lieberman, J. Biol.
Chem. 225:977,1957.

derivatives in the urine to be 20 alpha oriented.

Four new C-20 reduced (3 alpha, 5 beta ring A reduced)
metabolites of cortisone and cortisol have been reported.
These steroids are further reduction products of urocorti-
sol and urocortisone, usually determined in the urine as
indicators of cortisone and cortisol in the body. These
were namely, cortol, beta-cortol, cortolone and beta-
cortolone. This work was carried out by Fukushima.

D. K. Fukushima, N. S. Leeds, H. L. Bradlow, T. H.
Kritchevsky, M. B. Stokem and T. F. Gallagher, J. Biol.
Chem. 212:449,1955.

Schneider reports urocortisol is metabolized to the ¹⁶⁶

J. J. Schneider, M. Lewbart, P. Levitan and S. Lieberman, J.A.C.S. 77:4184, 1955.

urinary excretion product reduced at the C-20 in the beta epimer, namely, beta-cortolone.

Richardson has reported the occurrence of both of the

E. M. Richardson, J. Clin. Endo. and Met. 18:1399, 1958.

epimeric forms of cortisol in the urine of men receiving ACTH stimulation and the urine of a woman having panhypopituitarism who was receiving 400 mg of cortisone per day as replacement therapy.

Bulaschenko and co-workers have recently reported

H. Bulaschenko, Arch. Biochem. Biophys. 87:81, 1960.

three compounds with the glycol side chain and the ⁴-3-ketone group in ring A. These were reported as the 20 beta-OH reduction products of corticosterone and dihydrocorticosterone and the 20 alpha-OH reduction product of corticosterone. However, this last reported steroid is in error, as this laboratory synthesized this compound and our data do not agree with theirs.

Thus it can be seen that the majority of urinary metabolic end products have been reported to be oriented in the alpha form; however, an occasional report of beta epimers is to be found in the literature. One paper remains to be cited and that is the work of Lombardo and Hudson. They show that following C¹⁴-cortisone therapy

M. E. Lombardo and P. B. Hudson, J. Biol. Chem., 229:181, 1957.

to an adrenalectomized man, the major metabolite (9.5%) found in the urine was that of 20 beta epimer of the parent compound.

Thus any statement concerning the physiological significance of this investigation is conjectural. However, it is probably correct to state that the biological significance of C-6 hydroxylation and C-20 reduction is not understood and reports in the literature are conflicting. Is their presence related, either in a primary or secondary role, to the pathogenesis of hypertension, or do they represent the adrenal response to a substance produced by the hypertensive kidney, or yet, a response to blood pressure changes within the adrenal? In any case, this makes the problem of explaining why these steroids appear in the adrenal effluent of hypertensive dogs and fail to appear in the normotensive dogs most difficult.

As stated above, the biological activity of these ¹⁶⁸ hydroxylated and reduced compounds has not been determined. Abelson injected C¹⁴-cortisol and was able to isolate the

D. Abelson, F. Ulrich and C. N. H. Long, Proc. Soc. Exptl. Biol. and Med., 89:387,1955.

20 beta epimer of the parent compound from the blood of rats. When this compound was bioassayed in the adrenal-ectomized mouse for liver glycogen deposition, it was found to have an activity of about one-sixth that of cortisol.

Gleen studied the disappearance of the di-hydroxy-acetone side chain and the appearance of saturation in ring A by means of a liver enzyme preparation. Among others in a large series of compounds, 20 beta-ol-cortisol was used. This seems to show that the 20 reduction product is biologically active as far as the liver glycogen deposition test is concerned. The biological activity is not influenced by simultaneous administration of the 20 reduced compound.

E. M. Gleen, R. O. Stafford, S. C. Lyster and B. J. Bowman, Endocrinol., 61:129,1957.

Dorfman has synthesized 6 beta-ol-cortisol from 6 beta-ol-Compound S by incubating with a purified enzyme preparation of 11 beta-hydroxylase isolated from rat liver.

R. I. Dorfman and M. Hayano, Arch. Biochem. Biophys., 50:218, 1954.

Bioassay of the 6 beta-ol-cortisol showed it to be definitely less active than cortisol in the thymolytic assay, while the di-acetate of the compound was less than one-third that of cortisol acetate. It also is stated that when the electrolyte activity was checked, the 6 beta-ol compound was inactive.

The remainder of the 6-hydroxylated compounds that have been reported other than cortisol, are of interest in that their biological action, as modified by this substitution, may allow us to understand the significance of this group. Hayano reports that the biological activity of 6 beta-ol-

M. Hayano, M. Wiener and M. C. Lindberg, Fed. Proc., 12:216, 1953.

desoxycorticosterone is about equal to that of corticosterone, whereas the parent compound DOC is usually thought of as devoid of glycogenic activity.

Only two hydroxylases have been found common to both mammalian tissue and micro-organisms. These are the 6 beta and 11 beta hydroxylase systems. Murray and Peterson have reported that *Rhizopus arrhizus*, *Cunninghamella*

H. C. Murray and D. H. Peterson, U. S. Patent No. 2, 602,769, July 8, 1952; U. S. Patent No. 2, 672, 466, March 16, 1954.

blakesteena and Helicostylum piriforme all hydroxylate precursors at the C-6 site. Further, they report that the presence of a 6 beta-ol group inactivates ⁴-androstene-3,17-dione and adds glycogenic activity to desoxycorticosterone. Ogilvie and Hanze report that the presence of a 6-keto or 6 beta-ol group in progesterone type compounds,

R. B. Ogilvie and A. R. Hanze, U. S. Patent No. 2,671, 091, March 2, 1954.

which "demonstrates inhibitory properties in estrogenic, glucocorticoid, folliculoid, luteoid, testoid, hypertensive, salt retention as exhibited by DOC, spermatogenic and progestational activities."

Possible physiological significance of the findings

It would seem best to summarize the results of this work and to discuss the possible significance of this type of metabolic alteration.

In all of the following comparisons, in which t-tests

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were used, the requirement of homogeneous variance was met. The Bartlett test (1) for heterogeneity of variance yielded in all cases chi squares with probabilities greater than 0.01.

In Table 10, the blood pressure values in the experimental animals are all relatively the same; there is then no correlation between adrenal blood flow and the degree of blood pressure elevation in this group. This also holds true for the severity of hypertension and the increase in heart weight per Kg body weight because of the similarity (intergroup) of blood pressure and the small range (0.0515 - 0.0905 gm/Kg body weight, average of 0.0728) of heart weights in the experimental group.

For the data shown in Table 11, there is no significant difference in the weight of the adrenals/Kg body weight between the control and the experimental group.

For the data shown in Table 12, the mean adrenal plasma flow, in ml/hr/Kg body weight, for the control group is only slightly significantly larger (0.02 p 0.05) than that for the experimental group.

For the values shown in Table 13 and 14, the control and the experimental group means for the adrenal plasma flow, in ml/hr/gm of adrenal per Kg. body weight, were not significantly different (0.30 p 0.40).

1. The Bartlett test was carried out by Dr. Norma F. Besch

For the data shown in Table 16, the mean values for adrenal plasma aldosterone levels did differ significantly, ($t = 1.95, 0.05 \quad p = 0.10$).

It would seem of interest that Hartroft and coworkers

A. B. Eisenstein and P. M. Hartroft, *Endocrinol.*, 60: 634, 1957.

P. M. Hartroft and A. B. Eisenstein, *Endocrinol.*, 60: 641, 1957.

P. M. Hartroft, L. N. Newmark and J. A. Pitcock, *Hypertension*, p. 24, W. B. Saunders Co., Philadelphia, 1959.

have demonstrated that sodium-deficient diets in rats and dogs lead to an increased width of the zona glomerulosa, as well as a fall in serum sodium levels. Further, an increased production of aldosterone was obtained when these adrenals were incubated. This is clearly a homeostatic mechanism. The severity of the change of the zona glomerulosa increased with time and gave a significant regression curve. The controls remained relatively constant. One of the striking features of the adrenal histopathology was the hyperplasia of the glomerulosa cells.

These and other studies have demonstrated that hyperplasia of the zona glomerulosa results in an increased secretion of aldosterone by the adrenal gland. Perhaps, if our hypertensive sample was larger, we would have been able

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to demonstrate an even greater difference between the mean values of the two groups. However, we have good indication that an increase of aldosterone was present, since the sections of the adrenals did demonstrate marked hyperplasia of the zona glomerulosa. This would mean that the adrenal gland salt-saving mechanisms were operative, and thus perhaps the serum level of sodium was slightly increasing. This is clearly a pathogenic process. This increasing sodium level in the blood would, most likely, tend to raise the blood pressure, as in reverse, sodium restriction is a recognized treatment for hypertensive subjects. However, the exact mechanisms for the lowering of blood pressure by sodium restriction have never been clearly defined. These findings do not establish the existence of a causal relationship between a shift in osmolarity of body fluids and hypertension. Perhaps the suggestion of Sapirstein should be investigated, that is, to increase the consumption of tap

L. A. Sapirstein, Hypertension, p.276, W. B. Saunders Co., Philadelphia, 1959.

water in an attempt to dilute the hyperosmolarity of the body fluids. This would of course not correct the underlying mechanisms that are causing the increased secretion of aldosterone, but would increase the loss of sodium by the body.

It may also be argued that the operative procedure 174 used for the collection of adrenal vein plasma and the increased aldosterone concentration are related. It is certainly well recognized that a number of unrelated parameters can influence the secretory rate of aldosterone. Some of the more common factors that are reported to influence the increased secretion of aldosterone have been shown by Johnson, Leutscher, Lieberman, Farrell, Fine Bartter, Muller, Venning and Garcia-Llaurdo, along with their co-workers.

B. J. Johnson, A. H. Lieberman and P. J. Mulrow, J. Clin. Invest., 36:757,1957.

J. A. Leutscher, R.P.H.R., 12:175,1956.

A. H. Lieberman and J. A. Leutschner, Arch. Int. Med., 100:774,1957.

G. L. Farrell, R. S. Rosnagle and E. W. Rauschkolb, Circulation Res., 4:606,1956.

D. Fine, L. E. Meiselas and T. Auerbach, J. Clin. Invest., 37:232,1958.

F. C. Bartter, G. W. Liddle, L. E. Duncan, J. H. Barber and C. Delea, J. Clin. Invest., 35:1306,1956.

A. F. Muller and C. M. O'Connor, An International Symposium on Aldosterone, p.232, Churchill, London, 1958.

E. H. Venning, T. Primrose, L. C. S. Caligaris and I. Dyrenfurth, Metabolism, Clin. and Exper., 7:293,1958.

J. Garcia-Llaurado, Lancet I:1295,1955.

J. Garcia-Llaurado, Metabolism, Clin. and Exper., 6:
556,1957.

The most pronounced changes reported are due to electrolyte changes, blood loss, fluid volume changes, trauma and surgery. Therefore in this experiment, determination of aldosterone in adrenal plasma may fall into dispute due to the above mentioned factors.

However, the work of Davis and co-workers may eliminate partly this possibility, Davis was able to demonstrate that

J. O. Davis, M. J. Goodkind, M. M. Pechet and W. C. Ball, Am. J. Physiol. 187:45,1956.

when a transfusion is given to replace the blood collected from the adrenal vein, this protected the results from reflecting the changes due to bleeding. Since the controls underwent the same amount of trauma and surgery as did the experimental groups, the values determined in the controls were a reasonable basis for reporting changes in the experimental group. Further, an extremely interesting fact was reported. Only about one per cent of the adrenal vein aldosterone was ever recovered in the urine. Thus the factors regulating the metabolism and secretion of the

remaining 99 per cent of adrenal aldosterone secretion 176 remain obscure. Perhaps, the work of Ulick and Lieberman should be re-investigated. These workers report the pre-

S. Ulick and S. Lieberman, J.A.C.S., 79:6567,1957.

sence of tetrahydro-aldosterone in urine. From our knowledge of the urinary metabolic end products of the corticoids, where approximately 75 percent is secreted as the tetra-hydro-derivative, it would not be a far step to reason that aldosterone is likewise secreted in a reduced form and that the determination of the free, unconjugated, unreduced compound may not reflect the true aldosterone level in the urine.

As to the possible mechanism of action of aldosterone in its role in the pathogenesis of hypertension, no clear cut mechanism has been suggested. Nicholson in an elegant

T. F. Nicholson, Can. J. Biochem. and Physiol.,35: 641,1957.

experiment, has sought to localize the site of action of aldosterone on the kidney. He reports that he was able to damage the kidney tubules with mercuric chloride and that this did not alter the action of desoxycorticosterone and aldosterone, with respect to the action of these compounds

on sodium. Yet, damage to the proximal convoluted tubules,¹⁷⁷ by means of sodium tartrate, resulted in the prevention of the action of these compounds on the retention of sodium. Therefore, it is reasoned that aldosterone acts directly on the proximal convoluted tubules to cause an increased reabsorption of sodium. Thus if adrenal venous aldosterone were increased, either in a primary or secondary role to hypertension, it would certainly cause increased plasma levels of sodium and thus tend to assist the future progress of the disease.

Recently Farrell's laboratory reported that hyper-

J. B. Garst, N. P. Shumway, H. Schwartz and G. L. Farrell, J. Clin. Endo. and Met., 20:1351, 1960.

tensive patients fell into two groups; one group was comparable to the controls, while the second group of hypertensive patients was significantly different from the controls as far as urinary aldosterone was concerned. This increased aldosterone value was related to the mean diastolic pressure in the hypertensive patients. These findings confirm the work reported by Genest and co-workers. As

J. Genest, G. Lemieux, A. Davignon, E. Koiw and W. Nowaczynski, Science, 123:503, 1956.

J. Genest, E. Koiw, W. Nowaczynski and G. Leboeuf,

stated previously, this work of the Genest team has been criticized on the number of subjects and the lack of supporting clinical data.

Therefore, from the preceding discussion, it seems reasonable to assume that the increased aldosterone secretion played some role in furthering the development of hypertension in our experimental group.

From the data presented in Table 16, we can compute a daily dose for each group; by using the mean values of weight and adrenal aldosterone, the hypertensive dog is secreting about 604 micrograms per 24 hours, while the normotensive is receiving 216.5 micrograms per 24 hours. They received, on an hourly basis, 25.1 and 9.03 micrograms per hour for the hypertensive and normotensive dogs, respectively.

These values are well in keeping with the values reported by Davis for dogs with thoracic caval constriction,

J. O. Davis, M. M. Pechet, W. C. Ball and M. J. Goodkind, J. Clin. Invest., 36:689, 1957.

which ultimately developed ascites. Their reported value of aldosterone in the adrenal plasma was 560 micrograms per 24 hours or 23.4 micrograms per hour.

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Experimentally, there is disagreement as to the role of aldosterone in hypertension, Gross found that if aldosterone is given in large enough doses, along with salt, it will cause hypertension. Salt controls did not become hypertensive. However, in an earlier report they were

F. Gross, P. Loustalot and R. Meier, *Acta Endocrinol.*, 26:417, 1957.

F. Gross, P. Loustalot and R. Meier, *Experientia*, 11: 67, 1955.

unable to produce hypertension with a small dose. However, Kumar and co-workers reported doses as small as 1 microgram

D. Kumar, A. E. D. Hall, R. Nakashima and A. G. Gornall, *Can. J. Biochem. and Physiol.*, 35:113, 1957.

per day in rats, sensitized by unilateral nephrectomy and extra salt, resulted in hypertension.

The relationship between aldosterone and hypertension has led to speculation on the role of aldosterone in the pathogenesis of human hypertension. However, until methodological advancements for a simpler determination are made, no clear cut, definitive relationship will be established, since aldosterone is usually secreted in such quantities that its determination is extremely difficult.

Returning to Table 16, it will be noted that there is a significant difference between the adrenal plasma cortisol values in the two groups ($p < .01$).

This should, perhaps, have been an unexpected result; however, this type of reasoning cannot be supported by experimental data. By this it is meant that the response to stress by an out pouring of corticoids from the adrenal has been well substantiated in the past. Yet can it not be argued that hypertension constitutes a stress, even though all investigations have failed to demonstrate this difference. No reference could be found which states that hypertension per se constitutes a stress. In other words, does the increased work, exemplified by the myocardial hyperplasia, and increased peripheral resistance found in hypertension, constitute a chronic low level stress? Perhaps, some would argue that the acquisition of these pathophysiological states would be at such a slow rate that the subject would not be under stress.

Also some might argue that if a stress state existed in the hypertensive subject, why did the urinary studies not show this corticoid increased. This may be explained by several factors. If, as stated by Kornel, the liver enzymes that conjugate steroids are malfunctioning in the hyper-

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tensive subject, then the steroids would be reabsorbed by the kidney and placed in the general circulation once again. This would tend to increase the blood level, but again these would not be demonstrated by the usual analytical methods due to the fact that peripheral and visceral tissues would modify their structure and they would not react with the Porter-Silber reagent used for routine blood determinations. This is well documented and will be discussed in detail later. Also the biological effect of these altered compounds is not known, and they most likely will not suppress ACTH. Thus high levels of steroids which may potentially alter the normal physiology of the cardiovascular system would be found in the general circulation. Further, if ACTH was not suppressed by these compounds, it would continue to stimulate the adrenal, and thus produce a cycle of increased steroids in the blood.

No reference pertaining to ACTH levels in hypertension could be found. This type of determination would help to establish if the subject were under stress. The nodular cortical hyperplasia seen in the adrenal slides of the hypertensive dogs would indicate an increased ACTH stimulation of the adrenal, which would result, in turn, in an increased cortisol production.

In keeping with the above reasoning, Venning and co-workers found the excretion of conjugated steroids to be decreased in severe toxemia with hypertension as compared

E. H. Venning, B. Singer and C. A. Simpson, Am. J. Ob. and Gyn., 67:542,1954.

to toxemia without hypertension, This latter group had a higher level than normal, as both the free and conjugated forms.

Complete understanding of the finding of increased cortisol in the hypertensive dogs must await further research studies involving not only steroid determinations but also renal and liver function studies.

In Table 17, 18, 19 and 20, the data supporting the identification of six compounds from hypertensive and normotensive dogs is presented. It can be calculated that about five times more moles per liter of the BF steroids are found in the hypertensive dogs than in the normotensive group. The major fraction (about 32 per cent) of this BF area in the experimental group was found to be 20 beta-OH-cortisol.

Recknagel has been able to prepare in high purity

R. O. Recknagel, J. Biol. Chem., 227:273,1957.

a C-20-Keto reductase from rat liver; it will actively reduce the C-20 carbonyl of cortisone in the presence of a reduced triphosphopyridine nucleotide (TPNH)-generating

system. He states that the reduction exhibits a relative¹⁸³ specificity for TPNH, and is associated with the microsome fraction to which it is firmly bound. Also, it is relatively specific for steroids with the dihydroxy-acetone side chain, as two 17-deoxy type steroids were relatively inactive in the C-20 carbonyl reductase reaction.

The enzyme systems necessary for this type of C-20 carbonyl hydrogenation have not been described in adrenal tissue. Perhaps, a system similar to that for the C-11 carbonyl (cortisone) to C-11-ol (cortisol) reaction is supporting this occurrence.

The work of Hudson and Lombardo confirm the presence

P. B. Hudson and M. E. Lombardo, J. Clin. Endo. Met., 15:324, 1955.

of an enzyme system capable of this type of reduction. A human adrenal was removed and perfused with 17 alpha-hydroxyprogesterone. From the perfusate they were able to isolate 20 beta-OH-Reichstein's "S", among other compounds. Prior to the removal of the adrenal, venous blood was collected. They were able to isolate and partly identify two BF compounds; no name was given these compounds, but enough characteristics were listed to definitely state that the side chain was reduced (did not react with BTZ). Methanolic and sulfuric acid spectra indicated that the compounds were

substituted derivatives of either ring A and/or B. 184

In a similar study by Sweat of adrenal vein blood,

M. Sweat, J. Clin. Endo. Met., 15:1043, 1955.

he reports the possible presence of 20 beta-ol-cortisol. In both of these studies the patients received cortisone prior to surgery or ACTH prior and/or during surgery, in order to increase the amount of steroids present.

If a calculation is made of the total amount of steroid in the plasma extracted in the normotensive and hypertensive groups, they are found to contain 0.605×10^{-6} M/L and 3.15×10^{-6} M/L, respectively, in the BE areas.

In the following list are the compounds found in the hypertensive and normotensive groups:

Groups		Remarks on
Hypertensive	Normotensive	Identification
6 beta-ol-F	none	Tentative
20 beta-ol-THE	none	positive
THcortisol	THcortisol	positive
allo-THF	none	Tentative
THcortisone	THcortisone	positive
20 beta-ol-F	none	positive

In addition to the six compounds listed above for the hypertensive group, two other very polar compounds were

found. However, the quantity was far too small to be 185
identified with present methods.

Romanoff and co-workers identified the occurrence

E. B. Romanoff, P. B. Hudson and G. Pincus, J. Clin.
Endo. Met. 13:1546,1953.

of THcortisone and THcortisol in the adrenal vein blood
of a patient prior to adrenalectomy.

Still unexplained is the concentration of these
steroids in the adrenal vein plasma. If, perhaps, the
generalized vasoconstriction of the kidney extends to the
adrenal, then would this ischemia of some yet undetermined
factor, play a role in the production of these steroids?
Could this alter the biosynthetic pathway that the steroids
undergo during the hydroxylation in the gland? It might be
conjectured that the decreased plasma flow per hour per
gram of adrenal is a significant factor and alters the path-
way in the gland of the hypertensive dog. Of course, there
is no data to substantiate this.

If these types of hydroxylation alter the biological
activity, as far as the pituitary is concerned, i.e., the
glycol side chain would not suppress ACTH, then the adrenal
would be constantly under stimulation by ACTH to produce
more steroids. The cortisol values in the two groups were
shown to be different; however, the group with elevated

blood pressure did produce steroids, in addition to cortisol, which were atypical in concentration and structure. Then one must also propose that these bizarre steroids possess little or no glucocorticoid action, since no naturally occurring steroids have yet been demonstrated to possess dissociation of glycogenic and ACTH inhibitory action. In fact, those steroids devoid of glycogenic action are equally devoid of pituitary suppressing activity.

If the glycol side chain prevented the suppression of ACTH, then this may account for the chronic low level stimulation of the adrenal by ACTH and might have been the cause of the nodular cortical hyperplasia noted in the adrenal sections.

This chronic low level stimulation of the adrenal by ACTH might even, over a prolonged period of time, alter the type of steroid being produced. The Hechter group was able to show that chronic ACTH stimulation of the rabbit

E. Kass, O. Hechter, I. A. Macchi and T. W. Mou, Proc. Soc. Experl. Biol. Med., 85:583, 1954.

adrenal resulted in a change in the type of steroid produced. The rabbit normally secretes corticosterone into the blood, yet after ACTH stimulation the rabbit adrenal made 17 alpha-

Thus, perhaps the combination of all these factors contribute to the production and maintenance of a more severe form of hypertension. Perhaps aldosterone, like desoxycorticosterone, as reported by Gross, and salt will

F. Gross and P. Lichtlen, Arch. Exper. Path. u. Pharmakol., 233:323,1958.

increase sensitivity to epinephrine, norepinephrine, renin, hypertensin and vasopressin.

Closely related to the work of Kornel (cited above), is the work of Englert. He reports that in chronic renal

E. Englert, H. Brown, D. G. Willardson, S. Wallach and E. L. Simons, J. Clin. Endo. Met.,18:36,1958.

disease, the clearance of adrenal cortical steroids decreases in proportion to the severity of the disease. This again would account for increased biological half-life which results in prolonged action on the body; thus, this may account for the blood pressure changes seen in chronic renal disease, or at least may be a contributing factor.

Thus far, we have not been able to demonstrate any primary or secondary significance for the presence of these atypical steroids in the plasma of the hypertensive dogs.

Good evidence that these isolated compounds are being 188
formed by the adrenal and not re-circulated through the
adrenal blood is given by the work of Brown, Tyler and
Tamm. They were able to show that all reduced compounds

H. Brown, E. Englert, S. Wallach and E. L. Simons, J.
Clin. Endo. Met., 17:1191,1957.

F. H. Tayler, Am. J. Clin. Nutrition, 5:377,1957.

J. Tamm, I. Beckman and K. D. Voigt, Acta Endocrinol.,
27:292,1958.

in pooled human plasma were conjugated with glucuronic
acid. Since our extraction method would not remove steroids
that are conjugated, these atypical steroids must have been
formed to a greater extent in one pass through the adrenal
by the blood; yet some may have re-circulated as the free
compounds, but this is very unlikely.

The presence of the compounds reduced at C-20 would,
however, explain the non-detection of these steroids by
the usually employed Porter-Silber reagent, which has been
used to determine blood levels in normo-and hypertensive
patients. Also, this reduction would therefore make most
of the urinary steroids fail to react.

This is very similar to the type of steroids found
in the urine of patients with adrenal hyperplasia. Very
often the clinical picture does not agree with the

routine clinical laboratory report of steroids in the urine. Venning reported the presence of C-20 reduced

E. Venning, Guest Speaker, 1st Combined Meeting of the Canadian and American Assoc. of Clinical Chemist, Montreal, 1960.

compounds in the urine of patients with the hypertensive form of adrenal hyperplasia. These compounds accounted for the low values determined by the Porter-Silber reagent, which is specific for the dihydroxy-acetone side chain.

Recently, Neuman-Taylor of Western Reserve University stated that workers in their laboratory have found bizarre

A. E. Neuman-Taylor, personal conference, 1960.

hydroxylated steroids in the urine of hypertensive dogs. The exact nature of these steroids is as yet unknown. Perhaps, these are the urinary metabolic end products of the steroids we have found in the adrenal effluent of the hypertensive dogs in this study.

Thus it must be concluded that for the present, at least, no demonstrable clinical significance can be attached to these findings.

SUMMARY

This study was undertaken to determine whether or not an altered adrenal vein steroid profile existed in experimentally produced renal hypertensive dogs. These hypertensive dogs were compared to normal controls. This investigation was therefore primarily concerned with the isolation, identification and quantitation of adrenal venous steroids in these two groups of animals.

Standard recognized techniques of extraction, chromatography and classical chemical methods were used throughout the entire study.

A summary of the findings are listed below. In those instances that warranted statistical evaluation, the values obtained are also listed.

I. Biological Data

A. The following showed no correlation: a. adrenal blood flow and the degree of blood pressure elevation, b. severity of hypertension and heart weight, and c. the weight of the adrenals per Kg. body weight in the two groups.

B. The mean adrenal plasma flow, in ml/hr/Kg body weight, was larger (0.02 p 0.05) for the controls.

C. The means for adrenal plasma flow, in ml/hr/gm adrenal/Kg body weight, were not significantly different for the two groups (0.30 p 0.50).

D. The zona glomerulosa was markedly increased in

size in the hypertensive group.

II. Chemical Data

A. The mean values for adrenal plasma aldosterone levels were significantly ($0.05 > p > 0.001$) higher in the hypertensive group.

B. The mean values for adrenal plasma cortisol levels were significantly ($p > 0.01$) higher in the hypertensive group.

C. The more polar than cortisol area, BF, in the hypertensive group contained eight compounds. Four of these were identified as: 1) 20 beta-hydroxy-tetrahydrocortisone, 2) tetrahydrocortisol, 3) tetrahydrocortisone and 4) 20 beta-hydroxy-cortisol. Two were tentatively identified as: 1) 6 beta-hydroxy-cortisol and 2) allotetrahydrocortisol. Two very polar steroids were present in quantities far too small to identify.

D. On the other hand, only two compounds were found and identified in the BF area of the normotensive dogs: 1) tetrahydrocortisone and 2) tetrahydrocortisol.

E. The total molar quantity of steroids in the BF area, based on the molecular extinction coefficient, was 3.15×10^{-6} M/L of hypertensive dog plasma, while 0.605×10^{-6} M/L was found in the normotensive dog plasma.

In addition to a review of the literature relative to 6 beta-hydroxylation and C-20 reduction reactions, the

possible physiological importance of the significant findings are discussed in the light of relevant literature.

AUTOBIOGRAPHY

I, Paige K. Besch, was born in Bexar County, Texas, June 23, 1930. I received my secondary school education in the Lutheran schools of San Antonio, Texas, and my undergraduate training at Trinity University, which granted me the Bachelor of Science degree in 1954. At Ohio State University, I specialized in the Department of Physiology while completing the requirements for the degree Doctor of Philosophy. (For additional information see, American Men of Science, 10th Edition, 1960.)