RESERPINE AND CONVERSION OF STEROIDS BY RAT ADRENALS

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EFFECTS OF RESERPINE TREATMENT ON THE 'IN VITRO' UTILIZATION OF STEROID PRECURSORS BY RAT ADRENAL PREPARATIONS

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ABSTRACT

The findings in the present thesis are:

1. That reserpine affects the total body weight of rats as has been reported by other authors in various animal species.

2. That reserpine induced a hypertrophy of the adrenal gland, and that this hypertrophy was mostly due to an increase in cortical tissue.

3. That rat adrenal preparations from reserpine-treated animals produced a slight diminution in total utilization of progesterone-4-^{14}C as well as in incorporation into corticosterone when values were expressed on an equal weight basis. However, no statistical analysis could be carried out to ascertain the significance of these findings.

4. That treatment with reserpine induced a significant diminution in the *in vitro* utilization of cholesterol-4-^{14}C by rat adrenal homogenates.

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Abbreviations Used

Trivial Name	Systematic Name
18-OH-B	11β, 18, 21-trihydroxy-pregn-4-ene-3, 20-dione
19-OH-B	11β, 18, 21-trihydroxy-pregn-4-ene-3, 20-dione
Cortisol (F)	11β, 17α, 21-trihydroxy-pregn-4-ene-3, 20-dione
Aldosterone	11β, 21-dihydroxy-pregn-4-ene-3, 20 dione-18-al
Cortisone (E)	17α, 21-dihydroxy-pregn-4-ene-3, 11, 20-trione
19-OH-DOC	19, 21-dihydroxy-pregn-4-ene, 3, 20-dione
18-OH-DOC	18, 21-dihydroxy-pregn-4-ene-3, 20-dione
11-deoxy-cortisol (S)	17α, 21-dihydroxy-pregn-4-ene-3, 20-dione
Corticosterone (B)	11β, 21-dihydroxy-pregn-4-ene-3, 20-dione
11-dehydrocorticosterone (A)	21-hydroxy-pregn-4-ene-3, 11, 20-trione
11β-OH-progesterone	11β-hydroxy-pregn-4-ene-3, 20-dione
17α-OH-progesterone	17α-hydroxy-pregn-4-ene-3, 20-dione
11-deoxy-corticosterone (DOC)	21-hydroxy-pregn-4-ene-3, 20-dione
pregnenolone	3β-hydroxy-pregn-5-ene-20-one

Abbreviations Used (cont'd.)

Trivial Name	Systematic Name
progesterone	pregn-4-ene-3, 20-dione
Δ4-androstenedione	androst-4-ene-3, 17-dione
testosterone	17α-hydroxy-androst-4-ene-3-one
ACTH	adrenocorticotrophic hormone
G-6-P	glucose-6-phosphate
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
b.w.	body weight

I. REVIEW OF LITERATURE

INTRODUCTION

Several papers have been published during the last decade or so on the effects of reserpine on body weight, adrenal weight, catecholamine contents of the adrenal medulla, corticoid production and histological changes of the adrenal gland of the rat. Some conflicting data also has appeared, and it seems that many factors, for example the dose of reserpine given, the number of days of treatment, species difference and even the same species but different strain could be the cause of these contraversial reports. However, no comprehensive review of the literature has appeared regarding the effects of this Rauwolfia Alkaloid on the above mentioned parameters. The review of the literature in this thesis has been devided into two main chapters.

Chapter 1 deals with some general aspects of the two tissular components of the adrenal gland: the cortex and the medulla. The biosynthesis, enzymatic reactions and their *in vitro* requirements of the hormones elaborated by either tissue will be reviewed. Regarding the adrenal cortex some

considerations will be presented on the pituitary adrenal axis.

Chapter 2 will be devoted to the general pharmacological effects of reserpine, emphasizing on its action upon adrenocortical and adrenomedullary function.

CHAPTER 1

A. Rat Adrenal Glands

Adrenal glands of the rat are ovoid bodies situated on the upper pole of each kidney. Each gland is surrounded by a smooth connective tissue capsule. Immediately next to the capsule is the adrenal cortex, which surrounds the adrenal medulla lying in the centre.

Morphologically rat cortical parenchyma, like other mammalian cortex is arranged into three zones: the outer layer, where cells are arranged in regular groups called zona glomerulosa, the middle layer, where cells are comprised in more radial fashion called zona fasciculata and the innermost layer bordering the medulla which is called zona reticularis. This structural arrangement of adrenal cortex was first noted by Arnold in 1866. (1)

Embryologically the adrenal cortex develops from mesodermic coelomic epithelium tissue, and adrenal medulla originates from differentiated ectoderm-sympathetic nervous tissue.

Functional studies on the adrenal gland were initiated with the observation conducted in 1856 by Brown-Sequard who described fatal outcome of adrenalectomy in a number of

animal species, including the rat (2).

Swingle and Pfiffner in 1929 demonstrated that adrenalectomized animals can be maintained by injecting them with adrenal cortical extracts (3). In the early 1930's Reichstein in Switzerland and Kendall, Wintersteiner and Pfiffner in the United States isolated over 25 crystalline compounds from adrenal cortical extracts (4-6). Most of them were characterized as hydroxylated derivatives of perhydro-1, 2-cyclo pentenophenanthrene and of $\triangle$ 4 Pregnan series. Over 70 adrenal steroids have now been isolated or synthesized. Not all of these steroids are biologically active. Only few of them exhibit biological effects. On the basis of their biological effects in adrenalextomized rats adrenal cortical hormones or corticosteroids are divided into three groups: the "mineralo-corticoids" effect normal water and electrolyte balance, the "glucocorticoids" control disturbance in protein, carbohydrate and fat metabolism, and adrenal androgens having similar effects as male sex hormones (7).

B. Steroids produced by the Rat Adrenal Gland

Bush (8, 9) was the first to study steroid synthesized by the rat adrenal slices and secretion into the adrenal

vein blood, and showed that corticosterone was the major steroid produced. This was confirmed by Stack-Dunne (10) and numerous other investigators, both in studies carried out *in vivo* and *in vitro* (11-17). It has also been reported that corticosterone is produced both by zona glomerulosa (12, 18, 19) and zona fasciculata-reticularis (20, 21).

After discovery of aldosterone in 1954 by Simpson, Tait and their co-workers (22, 23), this compound has been isolated from rat adrenal vein blood (10) and shown to be synthesized by incubated rat adrenal glands (24). *In vitro* studies by Giroud et al (18) showed that aldosterone is exclusively produced by zona glomerulosa and not by the inner zones of the rat adrenal gland.

11-Deoxycorticosterone and 11-dehydrocorticosterone are also produced in the adrenal vein blood and incubation medium.

The other steroids produced by the rat adrenals have been identified as 18-hydroxylated compounds.

The first of these compounds was identified as the 18→20 cyclic hemiketal of 18-hydroxy, 11-deoxycorticosterone by Birmingham (25) and Péron (26). This compound is present in significant amounts in the adrenal vein blood of the rat (27).

It is interesting to note that this compound gives a typical Porter-Silber reaction (27-30) given usually by steroids bearing a dihydroxyacetone side-chain such as cortisol (31).

The second 18-hydroxylated compound identified by Péron (26) is the 18-20 hemiketal form of 18-hydroxy corticosterone.

The third of these compounds isolated from the rat incubation medium is believed to be a tautomeric form of 18-hydroxy corticosterone (32).

In vitro studies by Lucis et al (17) have resulted in the isolation of 19-hydroxy deoxycorticosterone from the rat adrenal gland using ^{14}C-progesterone or ^{14}C-deoxycorticosterone as substrate.

C. Lack of 17α-hydroxylase in the Rat Adrenal Glands

There are conflicting reports in the literature that whether or not the rat synthesizes 17α-hydroxylated steroids.

Existence of 17α-hydroxylated steroid has been reported in earlier works by Heard et al (77), Eisenstein (78) and Hoffman (28). Recently Brownell et al (79), using adrenal glands of hypertensive rats, in vitro studies showed that progesterone could be converted to 17α-hydroxy progesterone and compound S.

The criteria of identification was invariably based

upon positive Porter-Silber reaction and similar mobility with the authentic compounds in various chromatographic systems.

The possibility of existence of 17α-hydroxylated steroid has been ruled out by studies conducted by Birmingham et al (80), Ward and Birmingham (81) and recently by Laplante. (7).

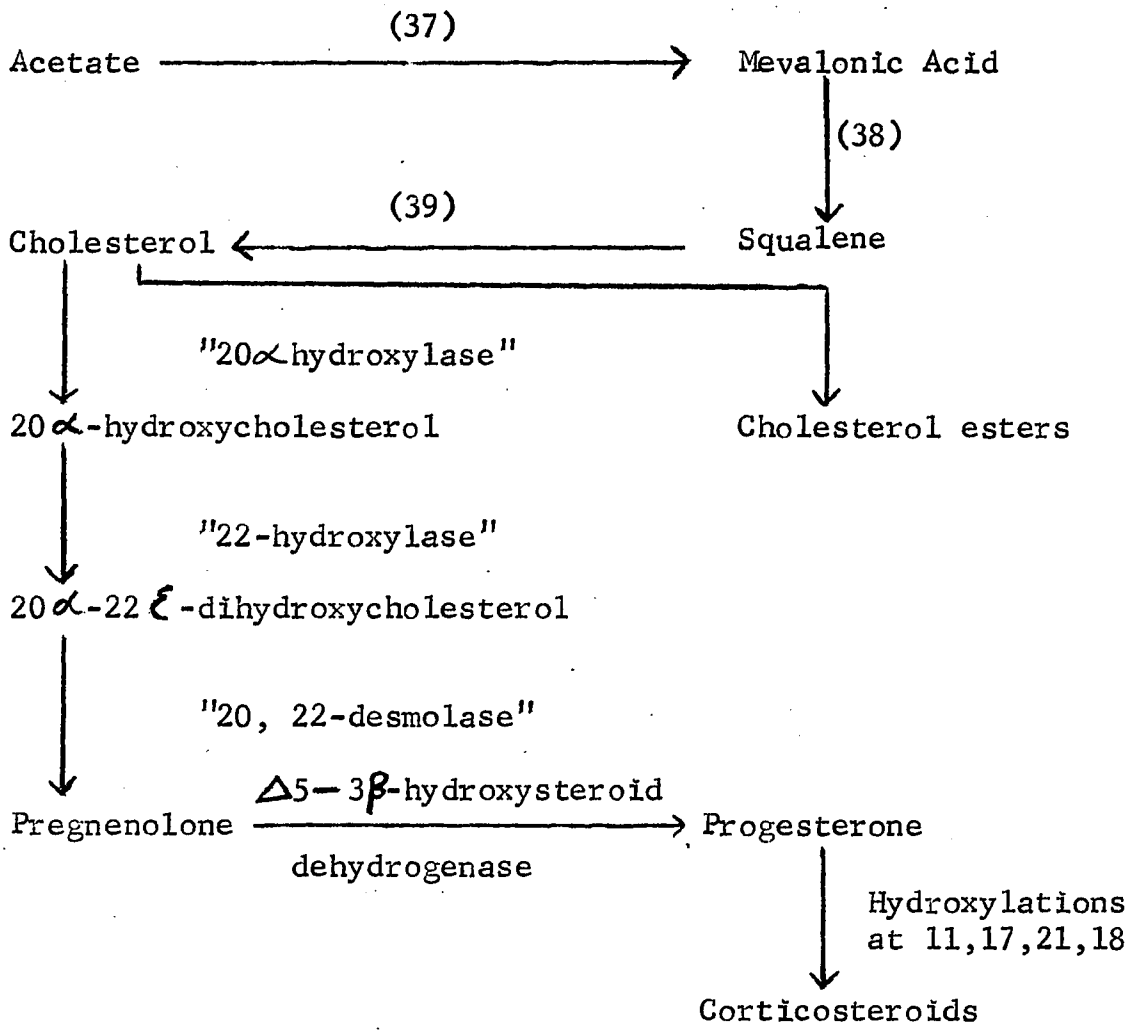
Earlier, Birmingham and Ward (25) and Ward and Birmingham (16) and Péron (26) isolated 18-20 hemiketal form of 18-OH-DOC from the rat adrenal glands. This compound reacts with Porter-Silber but does not reduce the tetrazolium chloride. Recently Dominguez (30) in vitro has isolated the same compound using ^{14}C-DOC as a precursor with rat adrenal glands. Two forms of this compound in reversible equilibrium have been designated compound M for the more polar and L for the less polar form.

D. Biosynthesis of Corticosteroids

The biosynthesis of adrenocortical hormones starts from acetate. Bloch (33, 34) in rat liver slices demonstrated that acetate is the principal if not the only carbon source of cholesterol. Still, the sequence of the reaction of the biogenesis of cholesterol in adrenal cortex is not fully known. At present, the most generally accepted scheme is based upon the classical studies conducted by Hechter et al (35)

with the perfused bovine adrenal gland. Grant (36) has reviewed the corticoidogenesis sequence, but still no significant change has occurred in the scheme presented by Hechter et al (35).

The major steps involved in corticosteroidogenesis relevant to this study will be reviewed briefly, and others are mentioned with references.



i) Side-Chain Cleavage of Cholesterol

Zaffaroni et al (40) first demonstrated conversion of cholesterol-^{14}C to corticosteroids in perfused bovine adrenal studies. In vitro studies conducted by Saba and Hechter (41) with cortical whole homogenates revealed that Mg^{++}, NAD and ATP were essential for the cleavage of cholesterol side-chain. Lynn et al (42) confirmed the report by the previous authors. Later studies conducted by Constantopoulos and Tchen (43) and Halkerston et al (44) with adrenal mitochondria claimed that NADPH and oxygen were absolute exogenous requirements for the side-chain scission of cholesterol molecule. These findings are in accordance with earlier reports, that ATP and NAD in the presence of Mg^{++} can operate as a NADPH generating system.

The mechanism of the side-chain cleavage of cholesterol is still not completely known. NADPH and oxygen are absolute requirements for the side-chain scission of the sterol molecule, suggesting that some kind of hydroxylating mechanism might be involved. Solomon et al (45) had isolated 20α-hydroxycholesterol from bovine adrenal homogenates incubated with labelled sterol. Later Shimizu et al (46) succeeded in isolating ^{14}C-isocaproic acid from 20 -OH-

cholesterol-22-^{14}C with the supernatant of bovine adrenal homogenates. Since this reaction required NADPH or an NADPH-generating system, it was suggested that another hydroxylation of side-chain occurred, possibly at carbon 22. Based on their further experiments, Schimizu et al (47, 48) put forward this hypothesis, that another hydroxylation at C-22 is the next step followed by the acting of a "desmolase" between carbons 20-22. These findings have been confirmed by Constantapoulos et al (49).

ii) Isomerization of Δ^5-3β-ol to Δ^4-3 keto

At least two operations are required for the conversion of pregnenolone to progesterone. These reactions are dehydrogenation of the 3β-hydroxyl group and shifting of Δ5 to Δ4. For these reactions at least two enzymes or enzymatic systems are involved, the Δ^5-3β-hydroxysteroid dehydrogenase (50) and isomerase (51). Actually these two enzymes or enzymatic systems are difficult to separate from each other, and are studied together under the name of Δ^5-3β-hydroxysteroid dehydrogenase. It is now known other steroid-producing tissues have this system, i.e. testis (50) and ovary (52).

Samuels et al (53) and Carballeira et al (54) reported

that the adrenal medulla also possess this enzyme.

Samuels et al (50) demonstrated that this enzymatic reaction is stimulated by NAD which acts as a hydrogen acceptor. This finding has been confirmed by Byer and Samuels (55) and Halkerston et al (44) from microsomal fraction of the beef adrenals. Also Halkerston et al (44) found that if this enzyme is dialysed, it loses its activity, and this could be restored by addition of NAD, Kowal et al (56. 57) with acetone preparation of corpus luteum and adrenal cortex demonstrated that NAD was the preferred co-factor as compared to NADP which had about 50% activity of the former. If NADH is added it inhibits this reaction, and this inhibition could be overcome by the addition of excessive amounts of NAD. Moreover, it is interesting to note that their preparation could not utilize cholesterol or 20-deoxy-pregnenolone, suggesting that an oxygen function at carbon 20 is necessary for the activity of Δ^5-3β-hydroxysteroid dehydrogenase.

Baille et al (58) in their histochemical studies reported that his enzyme seems to be mainly present in the zona glomerulosa. However, Levy and Rubin (59) have evidence that this is present in all zones of the adrenal cortex.

iii) Steroid Hydroxylation

Hydroxylation of the steroid molecule at various sites requires specific enzymes. For the hydroxylation at carbons 17, 21 and 11 beside specific enzymes, molecular oxygen and NADPH or NADPH-generating systems are required as co-factors. A brief survey of these hydroxylating enzymes and various requirements is given below:

a) 11β-hydroxylation

This enzyme, known as a trade mark of the adrenal cortex, has been studied most extensively and appears to be confined in all three zones of adrenal cortex. (60)

Recently, Carballeira et al (54) incubating bovine adrenal medulla with labelled steroid precursors showed that normal chromaffin tissue is also capable of 11β-hydroxylation. Possibility of this enzyme in the adrenal medulla was already discussed at Ciba Foundation colloquia on Endocrinology in 1953 (61).

Mitochondria is the site where the enzyme is localized (62, 63). A soluble fraction of 11β-hydroxylase has been prepared (64-66). Tomkins et al (65, 67) obtained three protein fractions from the calf adrenal acetone powder, and all of them were required for 11β-hydroxylase activity, beside

NADPH, oxygen and a heat stable co-factor obtained from the extracts of rabbit liver.

The adrenal gland is rich in ascorbic acid, about 400-500 mg/100 gram of fresh tissue. There is quite a conflicting data of the effects of this vitamin on 11β-hydroxylation. Hayano et al (68) reported that it produces 65% inhibition in washed residue of whole bovine homogenates, while Cooper and Rosenthal (69, 70) could not detect any significant effect on cell free preparation. The possible role of the ascorbic acid to adrenocorticoidogenis is still obscure.

b.) 17α-hydroxylation

17α-hydroxylase activity is associated with microsomal fraction (71). Using bovine adrenals Stachenko and Giroud in in vitro (20, 21) have reported that this enzyme is present only in zona fasciculatareticularis.

c) 21-hydroxylase

Giroud and Stachenko (20, 21) in in vitro studies, using bovine adrenal gland, showed that this enzyme is present in all zones of the cortex. Ryan and Engle (72) showed that this enzyme is located in "microsomal fraction" sedimented at 105,000 x g.

d) 18-hydroxylase

This enzyme is located in mitochondria of the adrenal cortex (73-75) and present in all zones of the adrenal gland (17, 76). The possible role of 18-hydroxylation to aldosterone either via 18-OH-B or 18-OH-DOC is still obscure.

iv) Oxygen and NADPH Requirement

Hayano and Dorfman (63, 83) first reported that oxygen is utilized by beef adrenal homogenates. Saffran and Bayliss (84) failed to produce corticoids if oxygen was replaced by nitrogen.

Later investigations carried out by Hayano et al (85-88) using $^{18}O_2$, D_2O and $H_2{}^{18}O$ gave a direct evidence that molecular oxygen is involved in 11β, 17α and 21 hydroxylations.

Requirement of NADP for 11β-hydroxylation in adrenal gland homogenates was demonstreated by Hayano and Dorfman (83). However, it was later shown that NADPH was the actual co-factor required for the hydroxylation (89). In the same study Sweat and Lipcomb pointed out that any intermediate of the Krebs' cycle could stimulate the hydroxylation, by acting as substrate for the generation of NADPH, for example

isocitrate + NADP ⟶ Oxalosuccinate + NADPH. This finding was confirmed by Grant (64).

Specific requirement of NADPH for hydroxylations at 11, 17, 21 and of the side-chain of cholesterol prior to cleavage, has been demonstrated (43, 44, 71, 72, 90). Also it is now well established that NADPH or NADPH generating system stimulates steroid production in vitro (91-93).

E. Adrenal Pituitary Axis

Smith in 1930 pointed out that adrenal glands of a hypophysectomized rat showed a marked decrease in size and weight (94). These changes could be reversed by daily transplantation of fresh pituitary tissue. Only the anterior portion of the pituitary gland could reverse the changes whereas the posterior part could not do so. The feed back relationship between adrenal cortex and pituitary was suggested by Ingle and Higgins (95, 96). The pituitary gland factor which was responsible for its action on the adrenal gland was isolated from protein fraction of pituitary extracts simultaneously by Sayers (97) and Li (98).

This factor called adrenocorticotropic hormone (ACTH) made it possible to study more specifically the stimulating effect on the adrenal cortex. When ACTH is administered the

adrenal gland weight increases along with various cytological, chemical or biochemical changes directly or indirectly associated with the effect of ACTH. The weight gain is entirely the result of hypertrophy of the cortical component, the medulla being little effected (100, 101).

The major effect of ACTH is on the stimulation of the cells of inner zones. However, there is little if at all change in zona glomerulosa (101-103). Both adrenal vein blood (104) and peripheral plasma (105) corticosterone concentration is increased in a manner which is linearly related to the log of the dose of ACTH injected. Apparently ACTH accelerates 20α, 22ξ-dihydroxycholesterol transformation to Δ5-pregnenolone by cleavage of the side-chain between C-21 and C-22 of cholesterol, liberating iso caproic aldehyde (106).

Mechanism of action of ACTH was proposed by Haynes and co-workers (107, 108) who proposed that it activates phosphorylase, and involves in accumulation of 3', 5'-adenosine monophosphate. This results in increased level of intracellular glucose-6-phosphate from the adrenal glycogen which by further metabolism through hexose monophosphate shunt generates increased amounts of NADPH necessary for steroid hormone hydroxylation. Péron (109) supported this theory showing

that rat adrenal glands *in vitro* maximally stimulated with ACTH, $NADH_2$ and 3', 5'-AMP produces same amounts of corticosteroids.

Apart from a direct action on steroidogenises, ACTH stimulates corticosteroid production through an adrenal weight maintaining action. It is now recognized that the hyperactivity of the adrenal cortex is secondary to an outpouring of ACTH by pituitary during non-specific stress conditions such as trauma, haemorrhage, exposure to cold and noxius agents (110).

F. Biosynthesis of Catecholamines

Catecholamines in adrenal glands are not the main issue of this thesis, still it was an interesting feature of our studies. A sketch and brief literature review of biosynthesis of these pressor amines is given as following:

1. Phenylalanine
2. Tyrosine
3. Dopa
4. Dopamine
5. Norepinephrine
6. Epinephrine

The chemical similarity between phenylalanine, tyrosine and epinephrine and the possible role of the two amino acids as precursors of catecholamines have been recognized for many years (111). Gurin and Delluva demonstrated the synthesis of epinephrine by injecting either ^{3}H-or ^{14}C-phenylalanine into rats and recovering, in vitro, radioactive epinephrine from adrenal glands (112). Goodall and Kirshner using bovine adrenal gland slices were able to demonstrate the formation of epinephrine, norepinephrine from radioactive tyrosine, dopa and dopamine (113).

i) Conversion of Phenylalanine to Tyrosine

Phenylalanine hydroxylating system was shown by Kaufman and co-workers in rat liver (114-117). Two enzymatic systems for complete reaction were also purified by these workers. The co-factors required for this reaction are NADPH and tetrahydropteridine. In the first reaction phenylalanine reacts with tetrahydropteridine and oxygen to give tyrosine, 5, 6, dihydropteridine and water. For the completion of reaction the second necessary enzyme reduces dihydropteridine in co-operation with NADPH and regenerates tetrahydropteridine which acts as co-factor again.

(1) Tetrahydropteridine + phenylalanine + O_2 ⟶ tyrosine + 5, 6, dihydropteridine + H_2O.

(2) 5, 6, dihydropteridine + NADPH + H^+ ⟶ Tetrahydropteridine + $NADP^+$.

A variety of pteridine derivatives, including tetrahydrofolic acid, can participate in this reaction.

Phenylalanine can be converted to tyrosine non-enzymatically in the presence of Fe^{++}, ascorbic acid ethylenediamine tetraacetic acid (EDTA). Fellman and Develin have shown conversion of phenylalanine to tyrosine in beef adrenal slices and degree of conversion was similar whether or not slices are denatured by boiling prior to incubation (118). This result indicates that a chemical reaction produces hydroxylation in adrenal tissue. Still it has to be determined whether such a system is operative <u>in vivo</u> or not.

ii) <u>Tyrosine to Dopa</u>

This reaction is least understood in the biosynthesis of norepinephrine and epinephrine. Rosenfeld et al have demonstrated the formation of radioactive dopa from labelled tyrosine in perfused adrenal gland (119). Also it has been shown that adrenal slices are capable of converting tyrosine into dopa (113, 120). Weiner has reported that homogenates of

adrenal medulla, boiled or unboiled, convert tyrosine into dopa in the same proportion (121).

iii) Dopamine from Dopa

Langemann using bovine adrenal medulla demonstrated the enzyme dopa-decarboxylase in adrenal medulla (122). Dopa-decarboxylase has been located in cell supernatant (122, 123). This enzyme has broad substrate specificity. Pyridoxal 5-phosphate is required as a co-factor for this reaction (124). Blaschko has shown that aromatic amino acids of L-configuration which possess an unsubstituted amino group and hydroxyl group on the aromatic ring in either 2 or 3 positions prove to be best substrate (123).

iv) The Conversion of Dopamine to Norepinephrine

Hagen, using radioactive dopamine with homogenates of chicken adrenal gland supplemented by several co-factors such as ATP, NAD and α-ketoglutarate, showed conversion to norepinephrine (125). The enzyme catalyzing the reaction is in the particulate fraction of adrenal medullary cells, mainly confined to chromaffin granules (126, 127). Levin et al in their solubilized enzyme preparation have been able to demonstrate that for each molecule of dopamine converted to norepinephrine, one molecule of ascorbate is oxidized to dehydroascorbate

and one molecule of oxygen is reduced to water (128).

Dopamine + ascorbate + O_2 $\longrightarrow$ norepinephrine + H_2O + dehydroascorbate.

This enzyme has a broad substrate specification.

v) Epinephrine from Norepinephrine

Bulbring has shown the conversion of norepinephrine into epinephrine using homogenates of dog adrenal gland supplemented with ATP (129). The methylating enzyme appears to be located in the cell supernatant (130, 131). Cantoni has shown that the active co-factor is s-adenosyl methionine (for the transfer of methyl group) formed enzymatically from L-methionine, and ATP in the presence of Mg^{++} (132). The enzyme phenylethanolamine-N-methyl transferase has broad substrate specification and is apparently localized almost exclusively in the adrenal medulla (133).

G. Other Aspects in Corticosteroid Biosynthesis

Here at this point it is felt to have a quick look into the steroidogenic aspects of the demedullated adrenal glands (regenerated adrenals) and of the adrenal medulla reported in literature.

The reports on corticosteroid secretion and production by the enucleated regenerated rat adrenal gland are quite

divergent. Holzbauer and Vogt (134) have reported that enucleated glands allowed to regenerate secrete normal quantities of corticosterone per unit tissue weight. Normal (135), abnormal (136, 137) and subnormal (138-142) levels of corticosterone in adrenal vein plasma of the enucleated and regenerated adrenal gland have been reported. Macchi et al (143) and Mason et al (142) have shown that regenerated rat adrenal glands produce normal levels of corticosterone. Elevated (139, 140, 144) and reduced (141) glandular corticoids levels have also been reported.

Birmingham et al (145) showed that total steroid output during two hours incubation period by enucleated glands was lower than that of normal, in both male and female rats. Brownie and Skelton (146), using progesterone-4-^{14}C as precursor incubated intact and regenerated rat adrenal homogenates and demonstrated relative lack of the 11β-hydroxylating enzyme in the latter.

Carballeira et al (54) have claimed that the bovine adrenal medulla has about 30% capacity that of the cortex to utilize all radioactive precursors studied except cholesterol.

CHAPTER 2

RESERPINE

A. General Aspects

The root of the plant Rauwolfia Serpentina has been used for centuries in native Indian medicine for the relief of a number of nervous conditions, including anxiety, excitement, manic psychoses and epilepsy. An active alkaloid, reserpine was isolated by Mueller et al (147) in 1952.

The action of reserpine, by whatever route it is administered, is characterized by a very slow onset and an unusually long period of effectiveness, sometimes five days. This has been found to be true for various species including monkeys, dogs, rabbits, cats, guinea pigs, rats and mice. About 10 - 20 minutes after administration of reserpine to the experimental animal, there may be a slight excitement and shivering, but, as time goes on, the animals become more and more quiescent and enter a state called tranquilization. Although they may be very quiet and inactive, the animals can be aroused at any time by various stimuli; most animals show signs of recovery after six to eight hours and, depending upon doses and species, all show normal behaviour after 24 - 72 hours.

One of the first signs that appear after reserpine administration is a pronounced miosis. It lowers blood pressure in both anaesthetized and unanaesthetized animals. There is no ganglionic and no adrenergic blockade.

B. Body Weight

Chronic treatment with the Rauwolfia Alkaloid produces retardation in the body weight.

Gaunt et al (148) in 1954 first pointed out loss in body weight of the rats after chronic treatment with the reserpine.

Eränkö et al (149) demonstrated that injecting young male rats with 0.04 mg/100 g b.w./day of reserpine for two months produces a highly significant (at 0.01% level) decrease in body weight. It has also been reported that treating male rats for 12 days with 0.025 mg/100 g b.w./day of reserpine causes retardation in growth only at the level of 1% (150). One could see that a significant level is dependent on the dose and number of days of treatment. However, Sackles et al (151) reported that reserpine given to male rats in the doses of 0.1 mg/100 g b.w./day for seven days and 0.007 mg/100 g b.w./day for fourteen days produces similar reduction in the body weight ($P < 0.01$). Another group of investigators

have reported that reserpine given at a dose of 0.025 mg/ 100 g b.w./day for six days does not produce any significant changes in the body weight of the rats (152).

Decrease in the body weight of the reserpinized cat (153) and of the reserpinized mouse (154) has also been reported.

C. Adrenal Gland Weight

Gaunt et al (148) in 1954 noted that a single injection of 0.01 mg/100 g b.w. of reserpine given to the rats, produces hypertrophy of the adrenal gland, suggesting that reserpine has a slight stimulating effect on the adrenal function. Since then several other authors have reported similar findings. However, data are quite conflicting, and it seems that beside many other factors, the dose of reserpine and the number of days of treatment could be the cause of these controversial reports.

Hertting and Horkicwicz (150) showed that daily injection of 0.025 mg/100 g b.w. for twelve days to male rats produced a significant ($P < 0.01$) increase in the adrenal gland weight. This increase was abolished by injecting animals 1.25 mg/100 g b.w. of cortisone along with reserpine. Giuliani et al (152) failed to demonstrate any significant change in

the adrenal gland weight of the rats, treated with 0.025 mg/100 g b.w. of reserpine for six days. However, a significant ($P < 0.005$) decrease occurred if 0.015 mg/100 g b.w. of dexamethasone was given along with reserpine.

Changes in the adrenal weight of the rat with different doses and days of treatment has been reported (151, 155). Studies conducted by Sackler et al (151) on rats (90 - 110 gram b.w.) with 0.1 mg of reserpine treatment for seven days showed that the weight of the adrenal glands was significantly increased ($P < 0.01$). However, with a daily dose of 0.007 mg of reserpine for seven days produced decrease in adrenal gland weight. Epstein et al (155) demonstrated that a small dose of 0.0025 mg/100 g b.w. of reserpine given to the rats for two days did not show any change in the adrenal gland, however, an increase in the adrenal gland weight was observed if the dose was increased to ten times.

Chronic treatment of the male rats with 0.04 mg/100 g b.w./day for two months did not result in any significant change in the adrenal gland weight (149). In this investigation animals were sacrified nine days after the last injection. Khazan et al (156) reported 30-50% hypertrophy in the adrenal glands of the rats after ten days of 0.001 mg/100 g b.w.

reserpine injection, but failed to observe any hypertrophy after 40 days of treatment. Another feature of this study was the main enlargement of zona fasciculata-reticularis.

Kitay et al (157) made the interesting observations that not only a single injection of reserpine (0.025 mg/100 g b.w.) but also epinephrine (0.02 or 0.04 mg/100 g b.w.) produces a similar significant ($P < 0.01$) increase in the adrenal gland of male Sherman rats. Similarly Halkerston et al (158) demonstrated that reserpine (two injections 0.25 mg on the first day and one injection of 0.125 mg on the second day) and ACTH (4 U.S.P. units on the first day and two injections of 4 U.S.P. on the second day) in Sprague-Dawley rats (135-150 g) produce a similar increase in the adrenal gland weight.

Various other investigators have reported increase in adrenal weight of the rat (159-161) and of the guinea pig (156) after reserpine treatment.

In the above-mentioned investigations it was suggested that the hypertrophy of the adrenal gland, caused by the Rauwolfia Alkaloid, is due to a stimulatory effect on the ACTH release which in turn affects adrenal glands.

D. Adrenocortical Function

The levels of corticosteroids in the plasma and in the

adrenal gland of reserpinized animals have been studied by various investigators.

Egdahl et al (162) in 1956 reported that a single intravenous injection of 0.02 mg/100 g b.w. of reserpine to the unanaesthetized dogs produces a marked increase in adrenal corticoid secretion. The highest values occurred between 1/2 and 3 hours after injection of the drug. It was also pointed out that the maximal corticosteroid values following reserpine administration are similar in order of magnitude to those obtained following the intravenous injection of large doses of ACTH, though comparatively much delayed.

In monkeys, a single dose of reserpine (0.1 mg/100 g b.w.) produces elevated levels of 17-OH-CS in the peripheral blood (163). Newcomer (164) has reported that a single injection (0.1 mg) of reserpine to 3 - 4 weeks old chicken produces increased concentration of free Δ^4-3-keto-corticosteroids (particularly corticosterone) in both the adrenal gland and plasma in 3 hours.

Elevated levels of corticoids in the plasma of the reserpine-treated rats also have been reported. Eechaute (165) in 1962 showed that either acute (0.5 mg/100 g b.w.) or

chronic (0.1 mg/100 g b.w. for 10 days) treatment of the rats with the Rauwolfia Alkaloid, produces increased levels of plasma corticosteroids. Also incubation studies with excised adrenals of the reserpinized rats showed increased levels of corticosteroid production. In the same report the non-inhibitory action of drug to block the adrenocortical response to acute cold stress (60 min. at $4^{o}C$) was also observed. Denti et al (161) reported that the corticosterone content of the whole gland in the female reserpinized rats (0.2 mg/100 g b.w., single injection) is higher ($P < 0.01$) than in control.

A recent report by an Italian group of workers (152) showed no significant changes in corticosterone levels both in plasma and adrenals of male Sprague-Dawley rats, treated with reserpine (0.025 mg/100 g b.w. for 6 days). However, if dexamethasone (0.015 mg/g b.w. for 6 days) was given along with reserpine, a decrease in adrenal corticosterone (from 5.6 µg/100 g to 3.8 µg/100 g) but no change in plasma corticosterone (from 12.7 µg/100 ml to 12.4 µg/100 ml) was observed.

Maickel et al (166) claimed that a single dose of reserpine (0.1 mg/100 g b.w.) given to male Sprague-Dawley rats produces a significant ($P < 0.02$) rise in plasma level of corticosterone (from 0.12 ug/ml to 0.42 ug/ml). Another interesting

feature of this study was that the drug given to adrenal-demedullated (regenerated adrenal gland) animals produced similar results as noted above. The authors failed to show any change in plasma corticosterone in hypophysectomized rats injected with reserpine. Cushman and Hilton (167) infusing hypophysectomized dogs with reserpine, did not observe any changes in plasma 17-OH-CS. Later, the same group reported that the injection of the drug given to the rat up to dose level 0.025 mg/100 g b.w., does not produce any significant rise in plasma corticosterone (168). The maximum increase (250%) was achieved with 0.1 mg/100 g b.w. of reserpine, and no further rise could be demonstrated, if the dose level is increased to fivefold.

Halkerston et al (158) recently demonstrated that the corticosteroid output of preincubated adrenal bisects obtained from male Sprague-Dawley rats incubated for 3 hours in the absence of steroidogenic agent was 22.4 µg/100 mg adrenal tissue for the untreated group and 18.4 µg/100 mg for the reserpinized group (two injections, 0.15 mg/100 g b.w. on the first day and two 0.075 mg/100 g b.w. on the second day). The addition of ACTH (250 mµ/ml) or 3'-5'AMP (15 mM) to the incubation media of adrenal bisects from the

drug-treated animals showed a significant increase (100%) in corticosteroid production as compared to untreated group. However, supplementing the incubation media with G-6-P (3 mg/ml) and NADP (2 mg/ml) did not enhance the corticosteroid output. Similar results were obtained if the Rauwolfia Alkaloid injection was replaced with ACTH (one injection of 4 U.S.P. units on the first day and two on the second day).

Increase in urinary 17-OH-CS both in monkeys (169) and human patients (156) have also been reported upon treatment with reserpine.

The increase in production of corticoids and the hypertrophy of the adrenal gland induced by reserpine have been attributed to the release of ACTH from pituitary gland. Thus, Kitay et al (157) in 1957 reported that a single injection of reserpine (0.25 mg/100 g b.w.) given to male rats reduces ($P < 0.01$) pituitary ACTH content. This was confirmed by Saffran and Vogt (170) and Maickel et al (166).

E. Depletion of Pressor Amines from the Adrenal Medulla and other Catecholamine-producing Tissues

Reserpine is an extremely potent agent with respect to its ability to deplete catecholamines from all the tissues of the body. A single dose is sufficient to produce a profound, prolonged depletion, although repeated small doses are more

efficient (171-178). Reserpine exerts both a central and a peripheral action on the sympathetic nervous system. There is an increase in central sympathetic activity as well as a direct depleting effect on peripheral tissues. The importance of the central effect and the peripheral effect on adrenal depletion varies with species (179) and same species but different strain (180). The section of the splanchnic nerves in cats and rabbits or the transsection of the spinal cord at T2 in rabbits prior to administration of a single injection of reserpine will markedly diminish the depletion of catecholamines from the adrenal gland (176, 181-185). However, the administration of reserpine to rats results in a severe depletion of or the release of catecholamines from the adrenal gland (182, 183, 186-188).

Introduction to the Present Study

As shown in the review of the literature, the enzymology of the adrenal cortex and of the adrenal medulla has been extensively investigated in each parenchyma independently. Few attempts have been undertaken to establish a possible functional relationship between these tissues which are intimately associated in most vertebrates. This association is more intriguing since various hydroxylases and similar coenzymes are involved in the biosynthesis of corticosteroids and catecholamines. In our laboratory, it has been possible to demonstrate that human chromaffin tumors (186a), and the normal adrenal medulla (54) from beef can effect various enzymatic reactions typical of the cortex when virtually pure preparations were incubated with labelled steroids. Thus, the capacity of normal chromaffin tissue to hydroxylate progesterone-4-^{14}C was found to be roughly 30% of that of the cortex. However, the same chromaffin preparations on equal weight basis, failed to utilize cholesterol-4-^{14}C. Also recently Wurtman (204) has postulated that the conversion of norepinephrine to epinephrine in the rat adrenal medulla is under ACTH control.

Reserpine as previously mentioned, is a powerful pharma-

cological agent, depleting the adrenal medulla (used also for other catecholamine producing tissues) of pressor amines. A concomitant depletion of the nucleotides of the medulla has also been reported (205). No studies have been published in our knowledge claiming that medullary enzymes are also discharged from the cells by reserpine. Since a prolonged depletion of catecholamines has been observed after reserpine treatment, it is possible that this drug might also interfere with catecholamine synthesis.

Halkerston et al (158) have reported that catecholamine depleted rat adrenal bisects produce decreased amounts of corticosteroids in vitro.

In vitro studies with adrenal glands in which adrenal medulla is absent (regenerated adrenals) have shown a deminished production of ultraviolet-light absorbing materials (145) and a marked impairment of 11β-hydroxylation in studies with progesterone-4-^{14}C (146).

The purpose of this study is to explore the effects of the administration of reserpine on the in vitro biosynthesis of corticosteroids by rat adrenal preparations. The present studies have been carried out with cholesterol-4-^{14}C and progesterone-4-^{14}C in an attempt to elucidate whether the inhibitory

action of reserpine occurs at the level of the enzymatic side-chain scission of cholesterol, the hydroxylating enzymes attacking progesterone or both.

A possible relationship between the findings with regenerated adrenals (145, 146), reserpinized adrenals (158) and the capacity of the medulla to hydroxylate progesterone (54) has been envisaged as a working hypothesis.

II MATERIALS AND METHODS

1. Serpasil

Serpasil, brand name of Reserpine, was obtained from Ciba Company Ltd., Dorval, P.Q. through the courtesy of Dr. W. Murphy. The active material was supplied in the form of a solution (2.5 mg/ml) in a vehicle consisting of a mixture of ethyl alcohol, propylene glycol and water (1:1:2). This vehicle without the active principle was also kindly supplied by Ciba Company and was used as a placebo in control groups of animals.

2. Rats

Male hooded rats (200 - 250 gram body weight) were obtained from Quebec Breeding Farm, Montreal. Twenty rats were obtained for each experiment. Upon arrival, animals were equally divided in four cages. Out of twenty rats ten were marked as group A or control and remaining ten rats as group B or experimental.

After two days both groups were weighed out separately. Subcutaneous injections were given to both groups for five days (unless otherwise mentioned) as following:

Group A: Each rat 0.05 ml of serpasil placebo.

Group B: Each rat 0.05 ml of serpasil (representing

0.125 mg of reserpine).

Animals were kept in the same room. Purina Chow food and water were given daily ad libitum.

On the sixth day after the first injection, rats were weighed out again. The animals were sacrificed by decapitation, adrenal glands excised, trimmed off surrounding fat and connective tissues and weighed out for each group..

3. Glassware

Clean glassware, free of radioactivity, was used throughout.

The glassware, in which radioactivity was used, was rinsed thoroughly with methanol and water. After that it was kept for two days in chromic acid solution and rinsed again for several hours with tap water. Finally it was rinsed with distilled water.

Vials (potassiumfree NO-SOL-VIT glass) in which aliquots of radioactivity were taken for counting, were used only once.

4. Buffer Solutions

Krebs-Ringer phosphate buffer (189), supplemented with fumarate (0.04M) and nicotinamide (0.04M) at pH 7.4 was used as homogenizing medium. Homogenates of rat adrenal glands were prepared in the cold room (approximately $4^{o}C$) in a tight-

fitting, all-glass apparatus. In the studies conducted with surviving adrenal quarters Krebs-Ringer bicarbonate buffer (189) containing 200 mg % glucose, (pH 7.4) was used.

5. Labelled Steroid Substrates

All radioactive steroids were purchased from New England Nuclear Corporation. The following substrates were used:

Cholesterol-4-^{14}C S.A. = 7.73 μg/μc

Progesterone-4-^{14}C S.A. = 6.84 μg/μc

Chromatography of these labelled materials resulted in a single radioactive band coinciding with the position of their corresponding authentic compound.

Aliquots of 2 μc of the precursors employed were pipetted into the incubation beakers and left overnight. The following day substrates were redissolved in 0.2 ml of propylene glycol. To these preparations either Krebs-Ringer glucose buffer (adrenal sections) or Krebs-Ringer phosphate buffer (homogenates) was added according to the desired experiment.

In certain experiments unlabelled progesterone was added in the amounts indicated in the experiments concerned.

6. Additives to Incubation

NADP and G-6-P as their sodium salts were obtained from Sigma Chemical Company. These chemicals were readily soluble

in Krebs-Ringer phosphate or Krebs-Ringer bicarbonate buffer at PH 7.4. They were prepared and added to the in vitro systems just prior to incubation. Unless stated otherwise, the following concentrations were used: NADP, 0.12 mM; G-6-P, 0.30 mM.

7. Conditions of Incubations

Incubation studies were carried out in a Dubnoff Metabolic apparatus using 95% O_2 and 5% CO_2 as gassing. Temperature of water bath was kept between 37 and 37.5°C. Incubation time was usually one hour. In certain experiments pointed out in 'Results' it was found necessary to shorten this incubation period.

8. Extraction of Steroids

At the end of the incubation period, 10 ml of ethyl acetate were added to each beaker to stop the reaction. Contents of the beaker were poured into a separatory funnel and extracted three times: twice with 70 ml of ethyl acetate, once with 70 ml of chloroform. All the extracts were pooled and washed with 20 ml of water. The extract was dehydrated over anhydrous sodium sulphate and filtered. The sodium sulphate was washed twice with 20 ml of a chloroform:ethyl acetate mixture (1:1), filtered and added to the previous filtrate. The total extract

was evaporated to dryness under vacuum at 50°C.

The dry residue was dissolved in 10 ml of methanol, and a 0.1 ml (if not otherwise mentioned) aliquot was taken for assay of radioactivity. The remainder was transferred quantitatively into a test tube and evaporated to dryness under nitrogen at 35°C. The crude, dry extract was ready for application to paper for chromatography.

9. Reagents

Methanol, ethanol (purified with 2,4 dinitrophenylhydrazine, three times distilled), benzene and acetone were all reagent grade (Fisher) and distilled prior to use.

Ethyl acetate (Fisher) and chloroform (Mallinckrodt) were also reagent grade and distilled 1 - 3 days before being used for extractions.

Absolute alcohol was used for making steroid standards and for determination of steroid hormones by ultraviolet light absorption method.

10. Preparation of Paper for Chromatography

Whatman No. 1 chromatography paper 57 x 46 cms was used. Seventeen cm wide strips were cut along the length. These strips (Batch of 16) were washed in a Soxhlet apparatus with methanol:benzene (1:1) for three days. After three days the

papers were hung to dry and stored in a paper folder. Before chromatography, the paper was cut lengthwise, making 2 - 4 cm wide strips at 1 cm distance. All the strips were 44 cm long and were joined by a common head 13 cm long. The application line, where extracts were applied, was 2 cm from the common head.

11. Application of Extracts on Papergrams

Crude steroid extract was quantitatively transferred to a test tube and evaporated to dryness at 50°C under a stream of nitrogen. The dry residue was dissolved in five drops of methanol: chloroform (1:1) and applied on the application line (2 cm from the common head) with the help of Wintrobe pipette. During application the solvent mixture was continuously evaporated under a dry stream of nitrogen. To make sure of the complete transfer of the residue, the whole procedure was repeated twice.

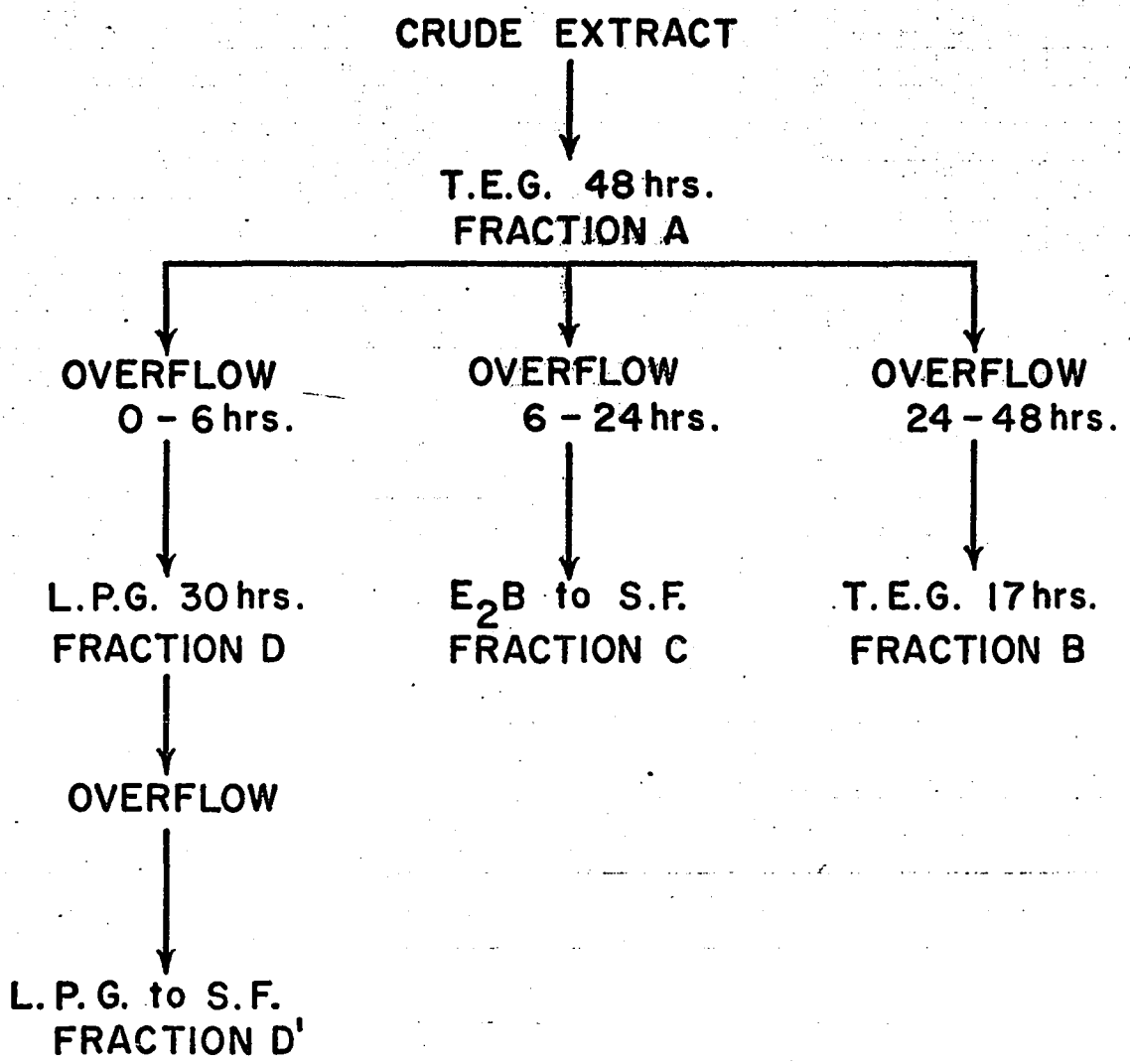
12. Paper Chromatography

Extracts were resolved by a slight modification paper chromatographic scheme developed in this laboratory (Figure I) (17, 189a).

Mainly solvent systems of Bush (190) and of Zaffaroni and Burton (191) were employed. An equilibration period is

Figure 1. Scheme of Serial Fractionation.

SERIAL FRACTIONATION



S.F. = SOLVENT FRONT

required before addition of the mobile phase in all Bush systems. In the latter systems impregnation in methanol/ethylene glycol (1:1) or methanol/propylene glycol (1:1) of the paper before application is necessary. After impregnation the paper was dried for three minutes in a fold of Whatman #2 paper, hung for three minutes in a fume cupboard, after which steroid residue was applied on the starting line. After application the paper was developed in a chromatography jar in a constant temperature room at 25°C and humidity 50%.

The systems used are listed as follows:

Bush A (190)

petroleum ether/methanol : water

100 / 80 : 20

Bush B5 (190)

benzene/methanol : water

100 50 : 50

Bush C (190)

toluene : ethyl acetate/MeoH : H_2O

90 : 10 / 50 : 50

D (192)

methylcyclohexane : toluene/methanol : water

100 : 25 / 80 : 20

E_2B (193)

Iso-Octane/t-butanol : water

100 / 50 : 90

B.F. (191)

Benzene/ formamide

L.P.G. (194)

Ligroin/propylene glycol

T.E.G. (195)

Toluene/ethylene glycol

T.P.G. (191)

Toluene/propylene glycol.

13. Detection of Steroids on Chromatogram

Each chromatogram, after development, was hung in a fume cupboard at room temperature until dry. Areas containing steroids were detected by

a)ultraviolet absorption

and/or

b) radioactive detection.

a) Ultraviolet Light Absorption

The paper strips were exposed to the ultraviolet light source using a Corning filter No. 9863. Steroids having an unsaturated Δ^4-3 keto group absorb ultraviolet light at 238 -

240 mμ. Kodak standard contact photographic paper was used to photograph ultraviolet light-absorbing spots.

b) Detection of Radioactivity

Radioactivity presented on papergrams was detected by (i) radioautography and/or (ii) radio papergram scanner.

i) Radioautography.

Dried paper strips were exposed to a No-screen Kodak X-rays film in a standard X-ray cassette. After three days the film was developed by standard X-ray film developing techniques. Usually about 3,000 c.p.m. could be detected.

ii) Radio papergram scanner.

The paper strips were scanned on a radiochromatogram scanner (Packard Model 7200) at appropriate sensivity and speed. Usually this technique was employed only for a rapid survey of the distribution of radioactivity on paperchromatograms.

14. Elution of Steroids from Paperstrips

30 ml syringes were used for the elution. Areas which showed radio-activity or ultraviolet absorption in paperstrips were cut out and hung with the needle directly into a round bottom flask. The strips were eluted twice with 30 ml of methanol. At the end the solvent was evaporated to dryness

under vacuum below 50°C.

In a few cases, where the radioactivity was spread over a wide area, the paper was cut into very small pieces and left overnight in methanol. The following day the methanol was filtered, the pieces of paper washed three times with methanol, and the pooled solvents were evaporated.

15. Assay of Radioactivity

Radioactivity of ^{14}C-labelled compounds was assayed in a Packard liquid scintillation spectrometer (models 3214 and 3375).

Aliquots of 1/100 to 1/20 of the total sample were used for the radioactive counts. These aliquots were pipetted out in the counting vials, completely dried under a stream of nitrogen and redissolved in 5 ml of scintillating solution. This solution contained 0.3% of 2.5 diphenyloxazole (P.P.O.) and 0.01% p-bis 1,2 (5-phenylozazolyl) 1-benzene (POPOP). The efficiency was 88% in both machines with 2% standard deviation.

16. Quantitative Determination of Steroid Hormones Ultraviolet Light Absorption Method

Steroid hormones with a Δ4-3 keto structure absorb ultraviolet light in the region of 240 mμ. The steroid is dissolved in ketone free ethanol, and the ultraviolet light

absorption spectrum is obtained in a Beckman DU spectrophotometer using silica cells. The quantity of the steroid hormone presented in the solution is calculated using the Beer-Lamberts law according to the following formula:

$$\mu g \,\Delta 4\text{-}3 \text{ ketosteroid} = \frac{\text{O.D.max} \times \text{M.W.} \times V \times 1000}{E}$$

O.D. = maximal optical density

M.W. = molecular weight of steroid

V = volume in milliliters

E = molar extinction coefficient of the steroid.

17. Chemical reactions of the Steroids

a) Acetylation

Steroids were acetylated according to the method of Bush (196). The dry steroid residue (30 - 60 µg) was dissolved in 0.1 ml dry pyridine, and 0.4 ml of acetic anhydride was added. The mixture was maintained at 60°C for 15 minutes and then evaporated to dryness under a stream of nitrogen. Twice 5 ml of methanol was added and evaporated to dryness. Finally 5 ml of dry benzene was added and evaporated to dryness. The dry mixture was directly applied on papergrams.

The 21-hydroxyl group of an α-ketol steroid is completely esterified under this condition. The 18-hydroxyl

group of aldosterone (hemiacetal form) is only esterified to about 15%.

b) Hydrolysis

Hydrolysis of steroid acetate was carried out by the modified method of Bush (197).

Dry steroid acetate (30-60 µg) was dissolved in 0.5 ml of methanol and 0.5 ml of sodium carbonate (2.5% aqueous solution). The mixture was kept at room temperature for 30 minutes and extracted with 2 x 10 ml of ethyl acetate. The pooled ethylacetate extract was washed with 2 ml of distilled water, dehydrated over anhydrous sodium sulfate, filtered and dried under a stream of nitrogen at 35°C. The dry residue was ready for chromatography.

c) Oxidation

Bush (198) method was employed for the oxidation of the 11-hydroxyl group to an 11-keto group. Dry steroid residue was dissolved in 0.5 ml of glacial acetic acid and 0.6 ml chronic acid (0.2% chromium trioxide) was added. The mixture was left in the dark for 20 minutes at room temperature. Under these conditions the 11β-hydroxyl group of C_{19} and C_{21} steroids are oxidized completely. At the end of 20 minutes 5 ml of distilled water was added and extracted with

2x10 ml of ethyl acetate. The organic phase was washed with 2 ml of saturated sodium bicarbonate, 2 ml of distilled water, dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was ready to be applied to the chromatogram.

d) Blue Tetrazolium (BT) Reaction

Steroid hormones with an α-ketol side-chain were measured qualitatively by the BT reaction described by Nowaczynski et al (199).

e) Porter-Silber Reaction

The Porter-Silber reaction is regarded as specific for steroids with a dihydroxy acetone side-chain (200). However, 18-OH-DOC also gives the same reaction with a maximum absorption at 400-410 mμ (25, 26), but the mechanism of the reaction involving the 18 - 20 cyclic hemiketal of 18-hydroxy-11-desoxycorticosterone is not understood.

Cupric acetate (0.005M in methanol) treatment of corticosterone and other non-dihydroxy side-chain steroid gives rise to glyoxal structure which yields a yellow chromogen with the Porter-Silber reagent (201).

Reagents:

Aldehyde free ethanol, freshly redistilled.

Dilute sulphuric acid (31 ml concentrated sulphuric acid to 19 ml distilled water).

Phenylhydrazine HCl, recrystallized from ethanol (16 mg phenylhydrazine in 10 ml dilute sulphuric acid, prepared immediately before use).

Method: The steroid residue and corresponding paper blanks were dissolved in 0.3 ml of ethanol and 0.45 ml of phenylhydrazinesulfuric acid solution. The tubes were stoppered, incubated in a water bath at 60^{o}C for one hour and then colled under tap water. The colour was read against paper blank in Beckman DK-2A recording spectrophotometer.

III RESULTS

All the results are expressed qualitatively or quantitatively. The body and adrenal gland's weights of the rats are given in grams and milligrams respectively.

The steroids isolated from incubation studies are expressed in terms of μg or in percentage conversion of the radioactive precursors. The percentage conversion is based upon the total radioactivity recovered from the incubation medium in the ethyl acetate-chloroform extract and not the radioactivity initially added.

Usually 85% or more of the radioactivity added is recovered using ethyl acetate-chloroform extraction (202).

Some of the results obtained were analysed statistically. Test of significance, i.e. "t test" was calculated as follows:

$$tc = \frac{\bar{d}}{\frac{sd}{\sqrt{n}}}$$

where tc = calculated value of t
$\bar{d}$ = mean of the differences of the control and the experimental

n = number of entries

sd = standard deviation of the difference and calculated as follows:

$$sd = \sqrt{\frac{\leq d^2}{n-1}}$$

where d^2 = sum of the squares of the differences between control and experimental

n = number of entries.

Standard deviation was calculated as follows:

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

where Y = deviate value

$\bar{Y}$ = mean value

n = number of entries.

Section A

In this section the changes occurred in the body and adrenal gland weight of the control (serpasil placebo-injected) and reserpinized (serpasil-injected) rats will be described. It should be emphasized here that the results obtained might not be the direct action of the placebo or the drug injected.

1. Body Weight of the Control Rats

Table No. I shows the body weight of the nine groups of the control rats. These groups of the rats were obtained during the period of September 1966 and May 1967. All the groups of the animals were similarly treated.

Two days after the arrival of the rats in the lab., the animals were weighed out individually and added together so as

TABLE I

Body Weight of the Control Rats after 5 Days

Group No.	Control 1st day x_1	Control 6th day x_2	$x_2-x_1=d$	d^2
1	1984*	2048*	54	4096
2	2396	2559	163	26569
3	2204	2294	90	8100
4	2346	2471	125	15625
5	2305	2343	38	1444
6	2315	2388	93	8694
7	2245	2305	50	2500
8	2225	2296	71	5041
9	2195	2229	34	1156
n=9	Mean=2246	Mean=2326	d=708	Σd^2=73225
			$\bar{d}$= 78.7	

Sd = 95.1

t_c = 2.47

t_8 at 5% = 2.31 $\quad t_c > t_8$ at 5%

2.47 > 2.31 $\quad P < 0.05$

*Total body weight of 10 rats

to obtain total body weight of the whole group. (Each group consisted of ten rats). This total body weight of the groups is shown under 1st day column. The same day 0.05 ml of serpasil placebo was injected to each rat of the group. Similar injections were given for next four consecutive days. Each injection was given 23-25 hours after the previous injection. Body-weight of the animals was not recorded during the five days' serpasil placebo treatment.

On the sixth day (24 hours after the last injection) the group of the rats was weighed out again and is listed under 6th day in the table.

During this period Purina Chow food and water was given daily,and always enough amounts were left for the night consumption.

From the data obtained it is clear that each group of the rats gained body weight. There was a wide range of gain of body weight of various groups of the rats from 34 grams to 163 grams. These results are in accordance with the report by Giuliani et al (152). Statistical analysis of the data showed significant ($P < 0.05$) increase in the body weight of nine groups of rats, treated with serpasil placebo for five days. Apparently the placebo injected does not effect the

normal growth and body weight of the rats.

2. Body Weight of the Rats after five Days of Reserpine Treatment

Table II shows nine groups of the rats treated with reserpine for five days. These groups were treated under exactly similar conditions as described previously for the control group except that serpasil, instead of serpasil placebo, was injected to the animals. About 0.075 mg/100 g b.w. of serpasil (about 0.05 ml solution) was injected to each rat of the group. This dose is ten times higher than the suggested dose by Schneider (203) for the chronic treatment of the rats. However, the choice of dose was in the same range as used by various other investigators (149, 151). About one hour after the first injection the rats showed sedation and reduction in spontaneous activity and shivering. After a few hours the animals became more and more quiescent. However, the animals were capable of normal reactions and were able to move around. The nictating membrane of the eyes were relaxed, and this state was continued until the next day. A pronounced loss of appetite was observed during this time as the animals did not consume any food or water provided.

The next day the rats showed normal behaviour, but again

TABLE II

Body Weight of the Rats after 5 Days of Reserpine Treatment

Group No.	Reserpinized 6th day x_1	Reserpinized 1st day x_2	$x_2-x_1=d$	d^2
1	1609*	1830*	221	48841
2	1887	2400	513	263169
3	1666	2282	616	379456
4	2000	2352	352	123904
5	1800	2292	492	242064
6	1901	2315	414	171396
7	1880	2250	370	136900
8	1628	2218	590	348100
9	1770	2200	430	184900
n=9	Mean=1794	Mean=2238	$d=3998$	$\leq d^2=1898730$

$Sd = 487$

$t_c = 2.74$

t_8 at 5% = 2.31 $\quad t_c > t_8$ at 5%

$2.47 > 2.31 \quad P < 0.05$

* Total body weight of 10 rats.

an injection of the Rauwolfia Alkaloid produced similar symptoms, as noted earlier. Besides, sometimes, some of the animals developed diarrhoea.

It is quite clear from the data that the animals lost body weight during the five days of reserpine treatment. The losses in the body weight of each group (ten rats) were between 10% to 30% of the original weights (first day weight). This decrease in the body weight is significant ($P < 0.05$). These results are in agreement with the findings of Eränkö et al (149), Hertting and Hornykiwicz (150) and Giuliani et al (152).

3. Adrenal Gland Weight of the Control and Reserpinized Rats

About 24 hours after the last injection, the animals were decapitated and operated upon in order to obtain the adrenal glands. Immediately after decapitation the control rats showed vigorous movements of the limbs, but the drug-treated animals did not show any movement whatsoever. Upon opening the abdomen, the intestine of the reserpinized rats showed marked dehydration and peculiar peristaltic movements. Kidneys were very shiny showing some enlargement. Adrenal glands of the drug treated animals were enlarged as compared to the control rats. Moreover they looked shiny and more pinkish.

Only one experiment was carried out where a single dose

TABLE III

Adrenal Weight (in mg) of Control and Reserpinized Rats.

Group No.	Control A	Reserpinized B	B-A=d	d^2
1	286*	368*	82	6724
2	304	382	78	6084
3	343	421	78	6084
4	373	446	73	5329
5	280	347	67	4489
6	344	421	77	5929
7	372	448	76	5776
8	378	445	67	4489
9	339	395	56	3136
10	387	470	83	6889
n=10	Mean=341	Mean=414	$\bar{d}$=73.7	Σd^2=54929

Sd = 78.10

t_c = 2.96

t_9 at 5% = 2.26 $\quad t_c > t_9$ at 5%

2.96 > 2.26 $\quad P < 0.05$

* Weight of 20 rat adrenal glands

(0.075/100 g b.w.) of reserpine was used, and the adrenal glands showed a mild hypertrophy. Increase in weight over the control group was about 10%. These results are in agreement with the reports by Kitay et al (157) who also pointed out a mild hypertrophy of the adrenal gland of the rat after a single injection of reserpine.

In all other experiments the treatment of the drug and the placebo was carried out for five days. The adrenal glands of the reserpinized rats showed about 16-22% increase in weight control. The data obtained from various groups are summarized in Table III. Statistical analysis of the data showed increase in the adrenal gland weight of the reserpinized animals is significant ($P < 0.05$). These results are in accordance with previous findings (148, 150, 151) but are not in agreement with the findings of Eränkö et al (149) who failed to observe any change in adrenal gland weight of the rats after two months of reserpine treatment.

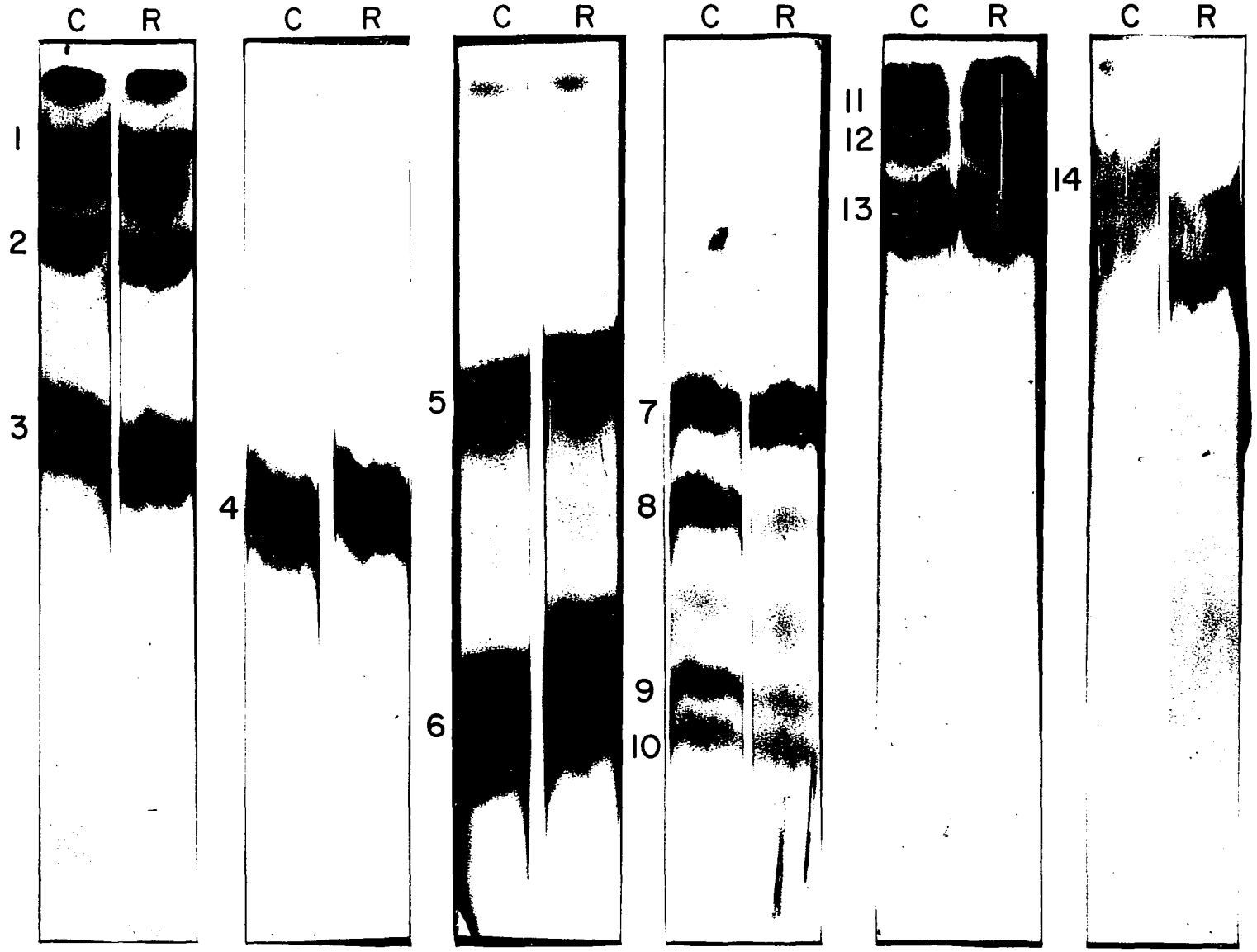
Section B

1. Conversion of Progesterone-4-^{14}C by Rat Adrenal Glands

The radioautograms shown in Figure 2 shows the labelled derivates of progesterone-4-^{14}C incubated with quarter adrenal glands of both control and reserpinized rats.

Figure 2. Conversion of Progesterone-4-^{14}C (2μc) by Adrenal Quarters of Control and Reserpinized Rats .

CONVERSION OF PROGESTERONE-4-^{14}C (2 μc)
BY ADRENAL QUARTERS OF CONTROL (C) AND RESERPINIZED (R) RATS



(A) T.E.G. 48 hrs. (B) T.E.G. 17 hrs. (C) B.F. 14 hrs. (C') E_2B 4 hrs. (D) L.P.G. 30 hrs. (D') L.P.G. 4 hrs.

In this experiment only a single injection of serpasil placebo and serpasil was given to the control and experimental rats respectively. 24 hours after, the adrenal glands were taken out, quartered and incubated in 10 ml Krebs-Ringer bicarbonate at pH 7.4 for one hour. Progesterone-4-^{14}C (2 μc) was used as a substrate. After extraction the dry residue was subjected to serial fractionation in paper chromatographic systems as shown on Figure No. 1, with the exception that the TEG 24 hours (Fraction C) overflow was applied to benzene-formamide system for 14 hours and overflow from this system applied to E_2B system as usual.

Fifteen radioactive zones were obtained and two zones, i.e.

zone # 6 corticosterone

zone # 13 deoxycorticosterone

were characterized (described later in the text). Another six radioactive zones were characterized tentatively (based upon their chromatographic mobility), and these are as following:

zone # 1 18-OH-B

zone # 2 19-OH-B

zone # 3 Aldosterone

zone # 4 19-OH-DOC

TABLE IV

Conversion of Progesterone-4-^{14}C by Rat Adrenal Glands

	Control		Experimental	
Adrenal Tissue	267 mg		300 mg	
Prog.-4-^{14}C	4.40×10^6cpm/13.2ug		4.40×10^6cpm/13.2ug	
Incubation Time	1 hour		1 hour	
	Inc. Medium	Tissue	Inc.Medium	Tissue
Extracted cpm	2.99×10^6	0.96×10^6	2.94×10^6	0.94×10^6
Recovered	75.70%	24.30%	75.76%	24.23%
Composition	%	%	%	%
18-OH-B*	1.02	2.26	1.33	2.08
19-OH-B*	1.02	-	1.64	-
Aldosterone	3.33	1.78	3.66	1.86
19-OH-DOC*	5.92	-	7.36	-
18-OH-DOC*	4.47	7.99	2.58	10.62
B*	30.04	16.46	25.34	27.43
A*	1.03	1.25	2.33	2.06
Zone #9	1.08	-	0.37	0.65
Zone #10	0.50	-	0.26	-
Zone #11	0.37	-	0.29	-
Zone #12	0.85	1.07	1.01	1.50
Zone #13	0.62	1.25	0.93	3.63
DOC*	12.52	3.03	11.89	2.52
Progesterone*	10.72	52.11	21.67	19.20

*Tentative identification

zone # 5 18-OH-DOC

zone # 7 compound A.

Table No. IV shows the quantitative distribution of the radioactivity in each zone. Total recovery of the radioactivity was 89% in the control and 88% in the experimental. The adrenal quarters of both control and reserpinized rats contained similar amounts of the radioactivity (about 24% of the recovered radioactivity). Conversion in incubation medium of the control was 62.77% and in that of experimental 59.71%. If these results are expressed in terms of 100 mg of the tissue, the corresponding values are 23.5% and 19.9%. In other words, on a unit weight basis the reserpinized adrenal glands showed about 18% lesser activity to utilize the exogenous labelled precursor as compared to the placebo-treated glands.

The results obtained with one day drug treatment of the rats were encouraging enough to continue further experiments. Another group of 20 rats was obtained, and half of them were treated with the placebo and the other half with reserpine for five days as described previously. The adrenal gland weight of the control group was 286 mg whereas serpasil-treated animals were 368 mg. After one hour incubation with 2 μc of progesterone-4-^{14}C the dry residue extract was subjected

TABLE V

Conversion of Progesterone-4-^{14}C by Rat Adrenal Glands

	Control		Experimental	
Adrenal Tissue	286 mg		368 mg	
Prog.-4-^{14}C	4.40×10^6 cpm/13.2ug		4.40×10^6 cpm/13.2ug	
Incubation Time	1 hour		1 hour	
	Inc. Medium	Tissue	Inc.Medium	Tissue
Extracted cpm	3.21×10^6	0.94×10^6	3.30×10^6	0.95×10^6
Recovered	77.55%	22.44%	77.56%	22.43%
Composition	%	%	%	%
Zone #1	0.60	-	0.39	-
18-OH-B*	6.40	2.14	3.09	1.61
19-OH-B*	1.00	-	0.42	-
Aldosterone*	6.12	2.53	2.81	1.39
19-OH-DOC*	0.59	-	0.58	-
18-OH-DOC*	5.59	2.68	10.90	5.04
A*	1.95	1.47	2.01	1.82
B*	24.47	16.02	27.93	19.19
Zone #9	1.40	2.00	2.29	2.98
Zone #10	0.95	0.95	0.57	0.99
Zone #11	0.55	0.53	0.39	0.66
Zone #12	0.42	-	0.52	-
Zone #13	0.52	0.61	0.49	1.18
DOC*	8.85	7.09	8.48	3.54
Progesterone*	16.80	33.31	16.33	31.89

*Tentative identification

to serial fractionation as described earlier. Table IV shows the percentage conversion of radioactivity in various zones. The percentage conversion by both control and reserpinized groups was of similar order as obtained in previous experiment. However, on a unit basis of adrenal tissue, the degree of conversion by the adrenal glands of reserpinized-treated rats was ± 31% lower than that of the control.

On a unit weight basis the adrenal tissue of the reserpinized rats in both these experiments retained less amount of radioactivity as compared to the controls.

In the foregoing experiments with adrenal quarters we have seen that the reserpinized adrenal glands had some impairment to utilize the exogenous labelled precursor. It was interesting to study, whether or not, the homogenized tissue shows the similar deficiency towards the added radioactive substrate.

Adrenal gland homogenates of the control (373 mg) and of the reserpinized (446 mg) rats were prepared separately in 10 ml of Krebs-Ringer phosphate buffer (pH 7.4) supplemented with fumarate and nicotinamide. These preparations were incubated with progesterone-4-^{14}C (2 μc), NADP and G-6-P for one hour at 37°C. The radioautograms of the serial

paper chromatographic fractionation showed twelve radioactive zones instead of usual fifteen obtained in studies with adrenal quarters. Moreover, the substrate added was practically exhausted, thus leaving no grounds for the comparison of enzymatic activity of the placebo and drug-treated adrenal glands.

To avoid the possibility of exhausting the labelled precursor added, in the next experiment the amount of tissue was reduced to about 1/5. Also 200 µg of the unlabelled progesterone were mixed with 1 µc of progesterone-4-^{14}C. The incubation time was reduced to fifteen minutes, and no co-factors were added. The radioautogram showed only eight radioactive zones. However, still some of the substrate added was left over. The per cent conversion by the adrenal glands of the placebo and the drug-treated glands are given as following:

% conversion of progesterone-4-^{14}C (1 µc+200 µg of unlabelled progesterone) per 100 mg of the adrenal tissue homogenates.

	Placebo-treated	Serpasil-treated
1.	87	75
2.	86	78
3.	88	76
4.	87	73

These results do not show a significant impairment of the adrenal glands of drug-treated rats, however, the tissue exhibited some deficiency to metabolize the exogenous labelled precursor.

2. Incubation with Cholesterol-4-^{14}C

Results obtained in previous sections lead us to examine the glands of the rats with some other substrates as well.

Since cholesterol is considered at the bottle neck of corticoidogenesis, it was interesting to study the transformation of this sterol by our preparations. Also, the use of exogenous labelled cholesterol might reveal some of the other enzymatic changes of the Rauwolfia Alkaloid-treated glands, that could not be demonstrated with radio progesterone (various steps involved from cholesterol to progesterone).

A series of experiments with cholesterol-4-^{14}C were carried out. Always homogenates of the adrenal glands were studied. No attempt was made to incubate the adrenal gland slices with labelled cholesterol. This was based upon the previous report by Carballeira et al (54) who consistently failed to catabolize the exogenous radio sterol with surviving bovine cortical slices under standard conditions of incubations.

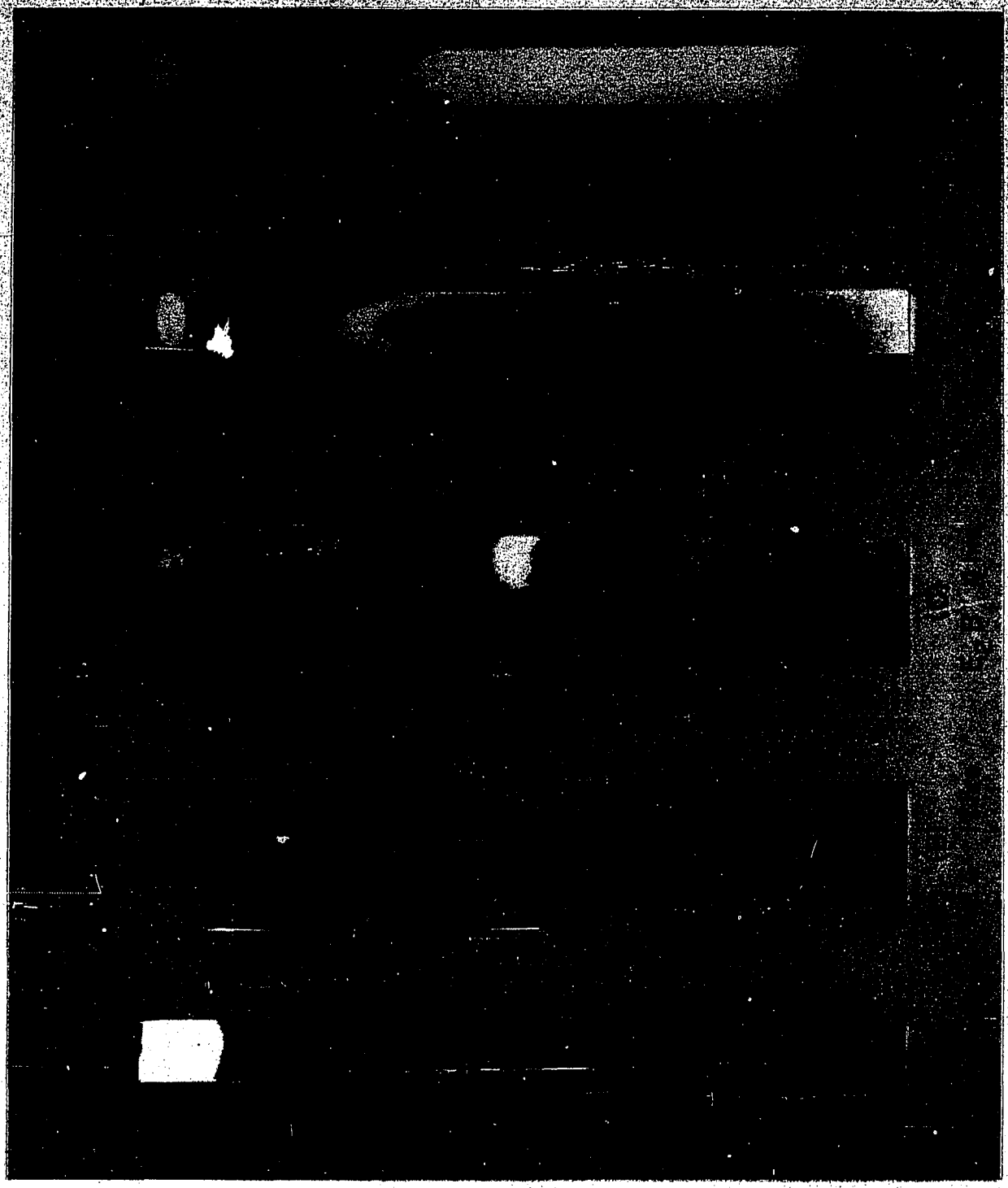
Adrenal gland tissue was homogenized in Krebs-Ringer

phosphate buffer supplemented with fumarate and nicotinamide. NADP and G-6-P were added just prior to the incubation. All incubations were carried out at 37°C for one hour. The extracts were subjected to serial paper chromatographic fractionation as shown in Figure 1.

Figure No. 3 shows the distribution of radioactive zones obtained by the placebo-treated rat adrenal gland homogenates incubated with cholesterol-4-^{14}C (2 μc). The ultraviolet photocopy (left) and radioautogram (right) of each fraction are placed side by side. Similarly Figure No. 4 shows the results of the drug-treated rat adrenal glands. There were nine ultraviolet positive and ten radioactive zones. Only zone No. 4 (corticosterone) and zone No. 8 (dexoxycorticosterone) were characterized (characterization shown at the end of the results).

The results obtained from the ten experiments are summarized in Table No. VI. The mean conversion of cholesterol-4-^{14}C by reserpinized-treated adrenal glands was 4.20% per 100 mg of the tissue, as compared to control value of 5.41% per 100 mg of the tissue. Analyzing the data statistically, it is shown that the reserpinized adrenal glands were significantly ($P < 0.05$) deficient to catabolize the exogenous

Figure 3. Conversion of Cholesterol-4-^{14}C (2μc) by Adrenal Homogenates of Control Rats.



CONVERSION OF CHOLESTEROL-4-^{14}C (2μc)
BY ADRENAL HOMOGENATES OF CONTROL RATS

(A) T. E. G. 48 hrs.

(B) T. E. G. 17 hrs.

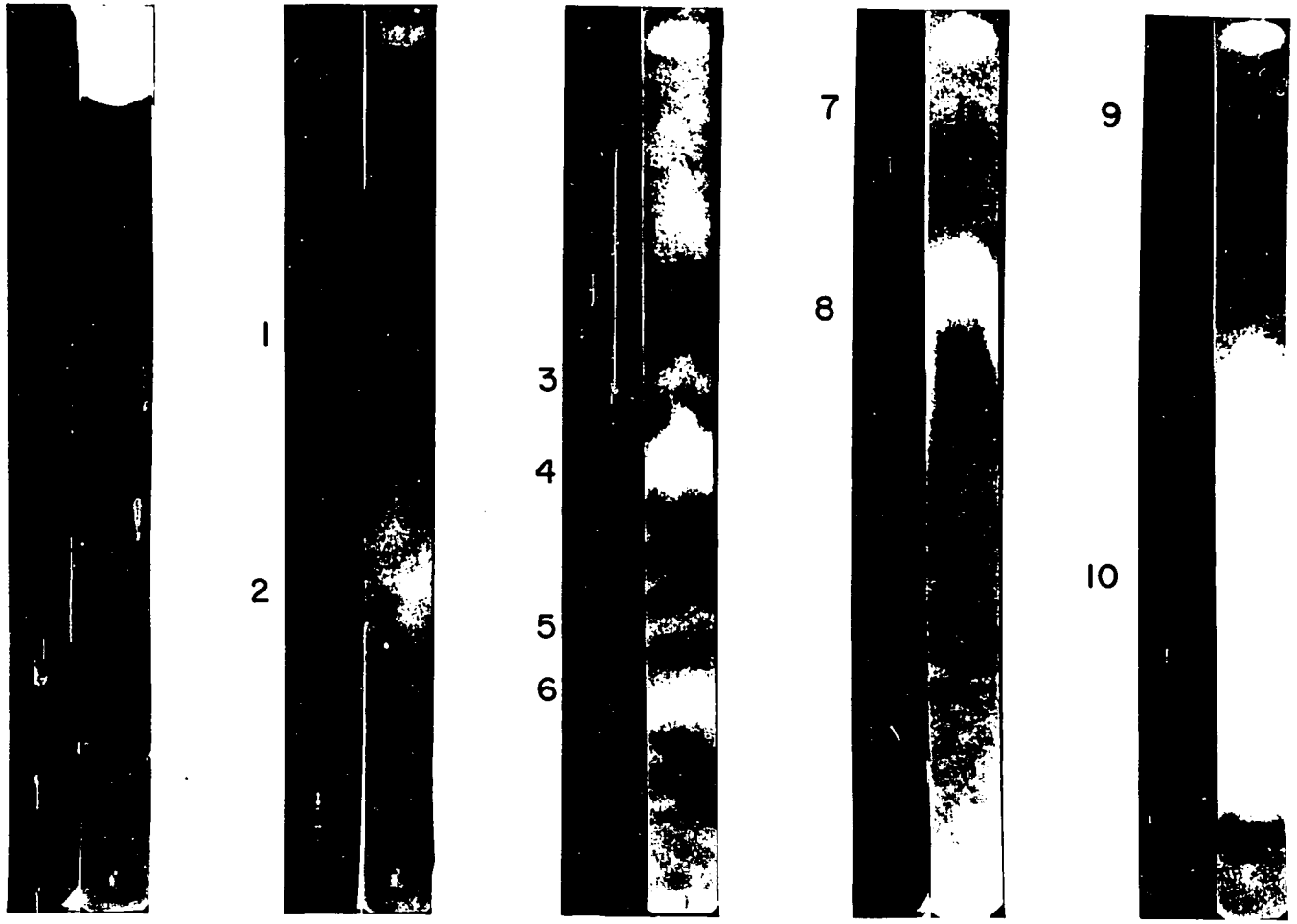
(C) E_2 B 4 hrs.

(D) L. P. G. 30 hrs.

(D') L. P. G. 4 hrs.

Figure 4. Conversion of Cholesterol-4-^{14}C (2μc) by Adrenal Homogenates of Reserpinized Rats.

CONVERSION OF CHOLESTEROL - 4 - ^{14}C (2μc)
BY ADRENAL HOMOGENATES OF RESERPINIZED RATS



(A) T. E. G. 48 hrs.

(B) T. E. G. 17 hrs.

(C) E_2 B 4 hrs.

(D) L. P. G. 30 hrs.

(D') L. P. G. 4 hrs.

TABLE VI

Percentage Conversion of Cholesterol-4-^{14}C by Rat Adrenal Homogenates

Group No.	Control A	Reserpinized B	A-B=d	d^2
1	3.45*	2.71*	0.74	0.5476
2	6.03	5.69	0.34	0.1156
3	3.71	3.43	0.28	0.0784
4	2.77	1.92	0.85	0.7225
5	4.05	3.49	0.56	0.3136
6	4.49	3.26	1.25	1.5625
7	8.40	5.63	2.77	7.6729
8	7.10	5.67	1.43	2.0449
9	8.03	5.24	2.79	7.7841
10	6.11	4.95	1.16	1.3456
n=10	Mean=5.41	Mean=4.20	d=12.17	Σd^2=22.1877
			$\bar{d}$= 1.217	

Sd = 1.568

t_c = 2.46

t_9 at 5% = 2.26 $\quad t_c > t_9$ at 5%

2.46 > 2.26 $\quad P < 0.05$

* Percentage conversion/100 mg tissue/hour

labelled sterol added. However, it was difficult to point out the steps which were impaired.

In another experiment where NADP and G-6-P were omitted, the conversion per 100 mg of the adrenal gland tissue homogenates by reserpinized rats (2.79%) was lower than that of the placebo-treated rat adrenals (3.54%). Another interesting feature of this experiment was the amount of corticosterone found as measured by ultraviolet-light absorption. Values for the control study (19 μg/100 mg of the adrenal tissue) were higher than those of experimental (14 μg/100 mg of the adrenal tissue). On the other hand, the measurement of DOC by the same method revealed a greater accumulation of DOC in the experimental (19 μg/100 mg of the adrenal tissue) than the control (16 μg/100 mg of the adrenal tissue) thus suggesting certain impairment in 11β-hydroxylation.

Halkerston et al (158) also showed that the corticosterone production by reserpinized adrenal glands was lower than the control value.

Table No. VII shows the per cent conversion of the cholesterol-4-^{14}C in labelled corticosterone by the rat adrenal homogenates. Statistical analysis of the data did not show any significant decrease in labelled corticosterone by the reserpinized group. However, it can be seen that

TABLE VII

Conversion of Cholesterol-4-^{14}C to Corticosterone

by Rat Adrenal Homogenates

Group No.	Control A	Reserpinized B
1	1.23*	1.21*
2	3.40	2.99
3	1.47	1.15
4	3.15	2.58
5	2.72	2.02
6	4.94	3.09
7	4.09	3.19
	Mean = 3.00	Mean = 2.32

* Percentage conversion/100 mg tissue/hour.

mean value for the control group is 3.00 as compared to the 2.32 for the reserpinized group. The Rauwolfia Alkaloid treated adrenal glands of the rats are about 29% deficient as compared to the control to metabolize the exogenous labelled precursor to corticosterone.

Section C

Characterization of Steroids

1. Corticosterone

This compound was isolated in all of our experiments and showed ultraviolet absorption maximum at 240 mμ.

i) Sulphuric Acid Spectra

The spectrum in sulphuric acid of the isolated corticosterone, both from control and reserpinized rat adrenal glands, was identical with that of authentic corticosterone (Figure 5).

ii) Acetylation

Pooled corticosterone from various experiments was applied on E_2B as shown on Figure No. 6. The distribution of five strips is of the following order: The first strip represents the running rate of authentic compounds. The first pair represents the ultraviolet photocopy and radioautogram of the control experiment. The second pair represents the

Figure 5. Sulphuric Acid Spectra of Isolated Corticosterone.

SULPHURIC ACID CHROMOGEN SPECTRA

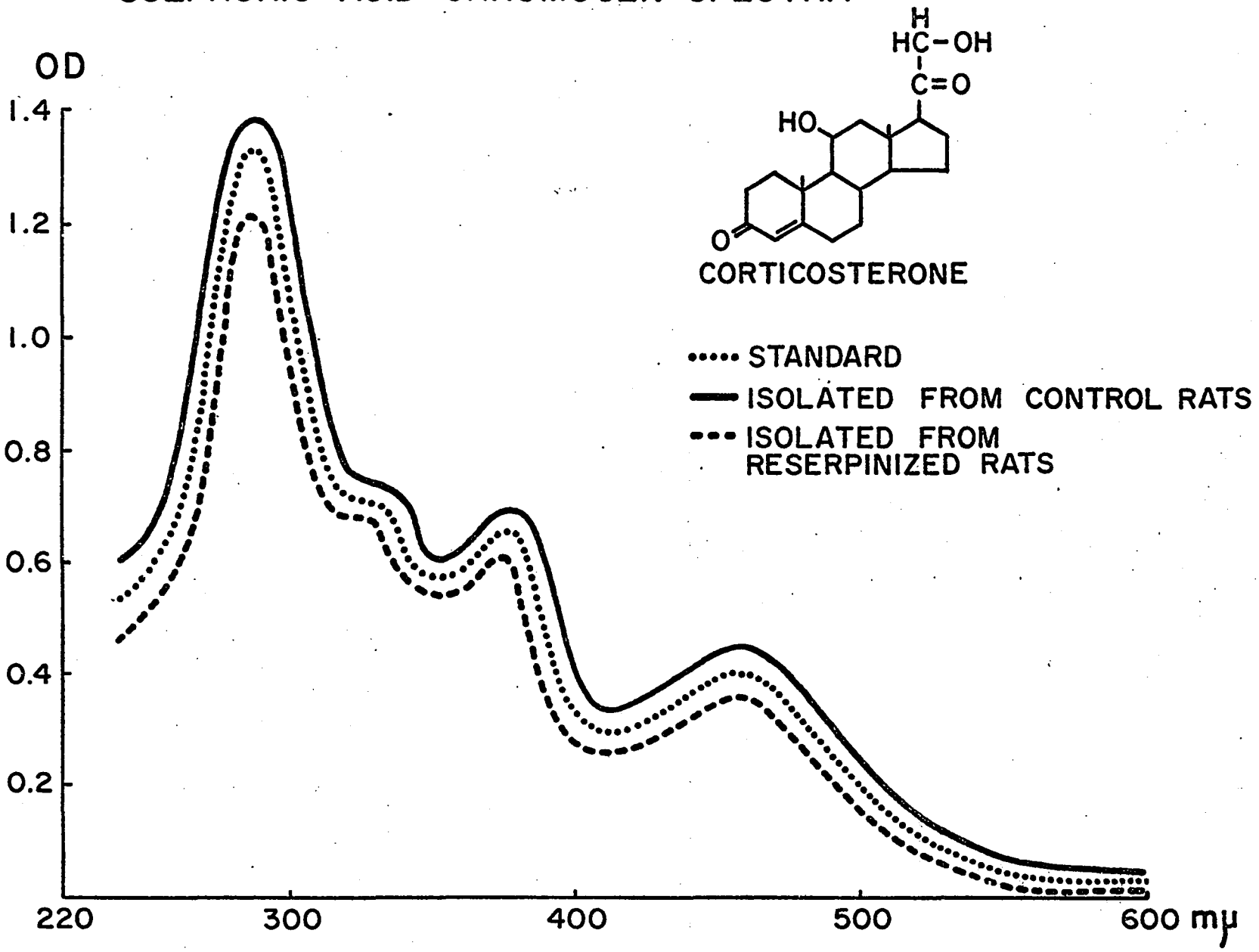
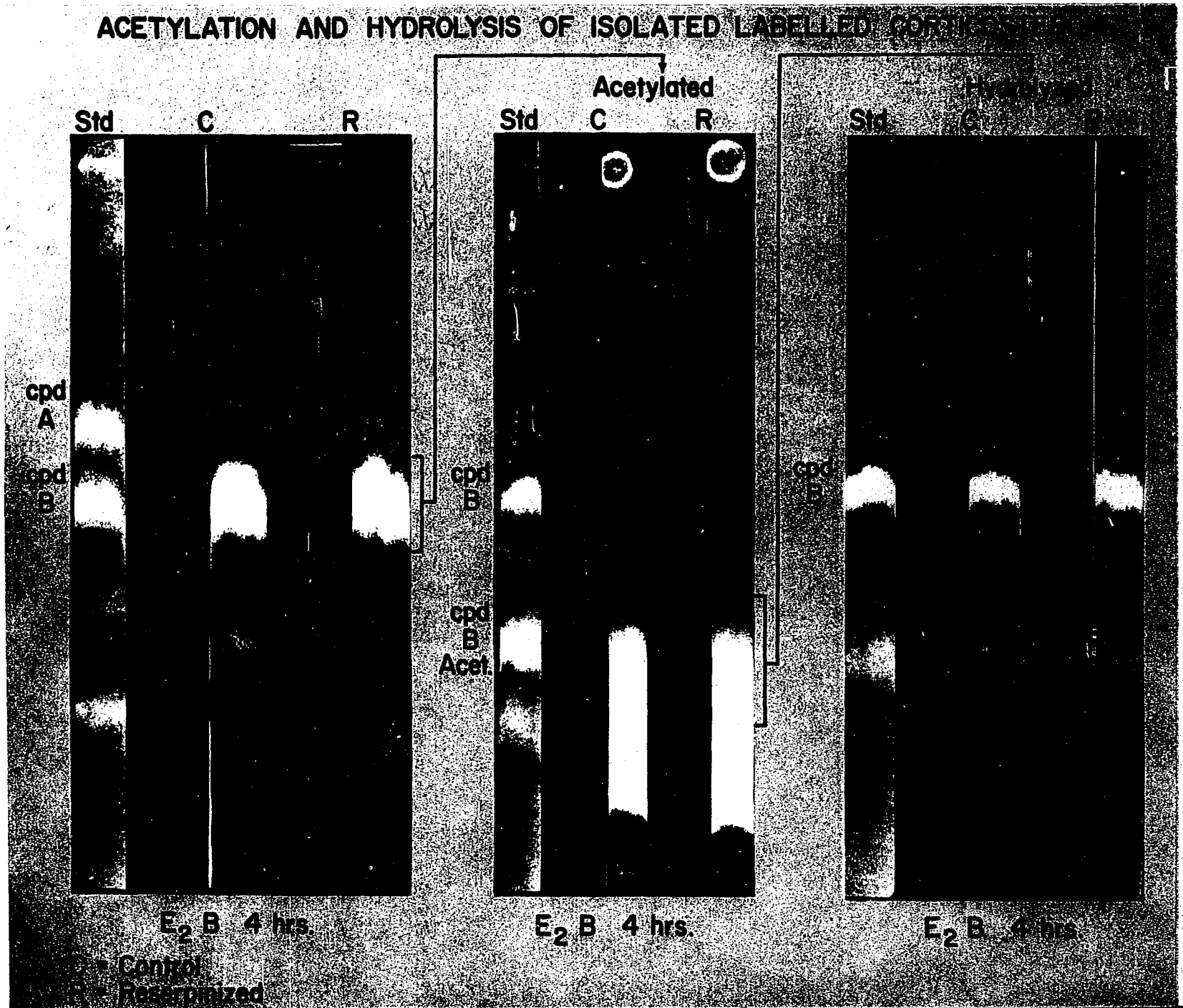
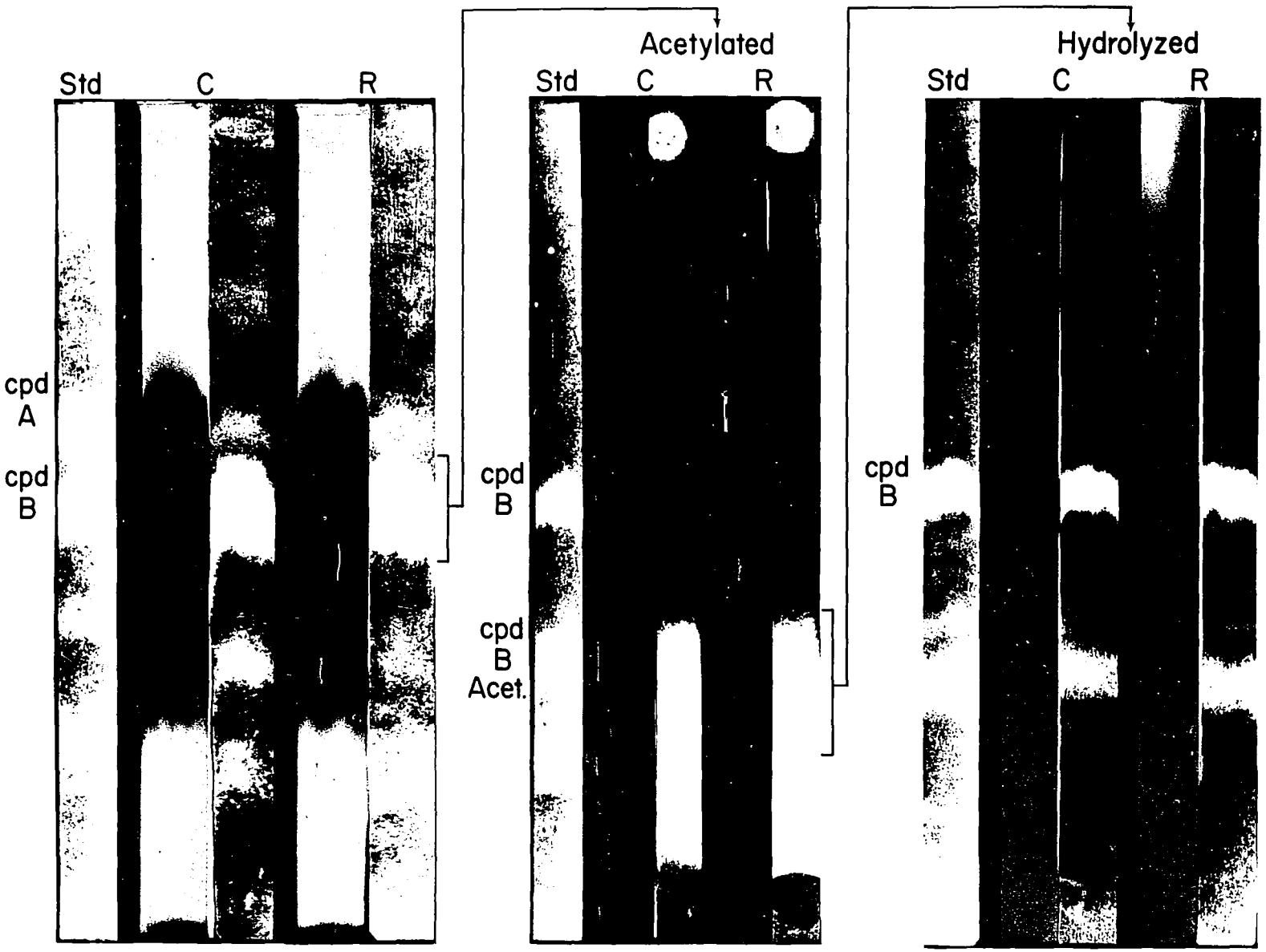


Figure 6. Acetylation and Hydrolysis of Isolated Labelled Corticosterone.


ACETYLATION AND HYDROLYSIS OF ISOLATED LABELLED CORTICOSTEROIDS
Acetylated
Std
C
R
Std
C
R
Std
C
R
cpd A
cpd B
cpd B
cpd B Acet.
cpd B
E2 B 4 hrs.
E2 B 4 hrs.
E2 B 4 hrs.
C = Control
R = Reserpinized

ACETYLATION AND HYDROLYSIS OF ISOLATED LABELLED CORTICOSTERONE



C = Control
R = Reserpinized

ultraviolet photocopy of the experimental. A similar type of arrangement is presented in Figure No. 6.

The zone corresponding to the authentic corticosterone was cut, eluted and dried. Part of the dry residue was subjected to fast acetylation according to the method described by Bush (196). The mixture was applied on E_2B system. Corticosterone isolated and acetylated showed similar running rate as the authentic B-Acetate. On hydrolysis the original compound was isolated and showed similar chromatographic mobility as the authentic corticosterone E_2B system.

iii) Oxidation

On oxidation of isolated corticosterone with CrO_3, the dehydrocorticosterone yielded on chromatography in the Bush B5 system a single radioactive zone with the same mobility as authentic dehydrocorticosterone. This zone on acetylation at 60°C yielded compound A Acetate which had the same chromatographic mobility in the TPG system as the standard (Figure 7).

2. Deoxycorticosterone

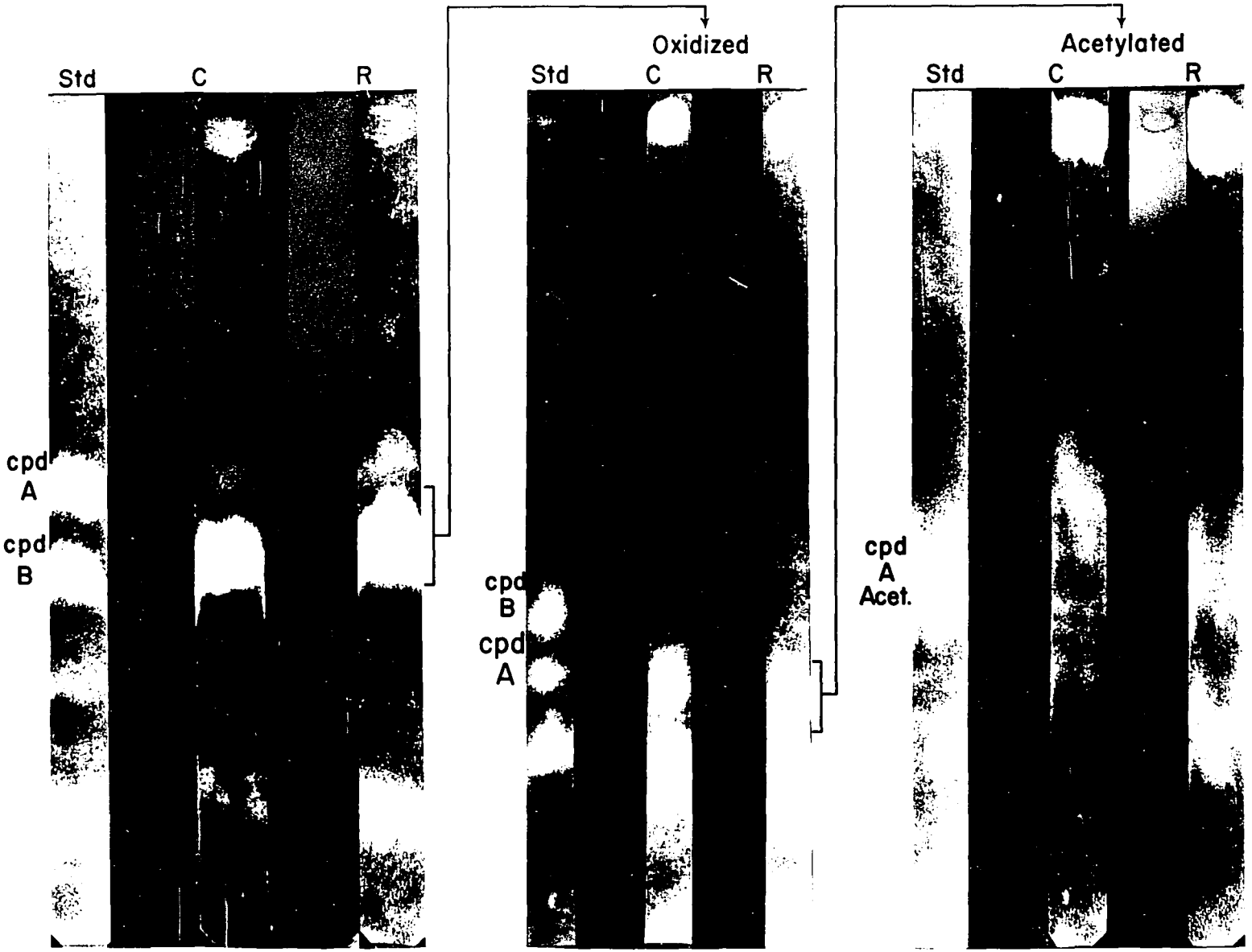
This steroid, isolated both from control and experimenal, showed ultraviolet absorption maximum at 240 mμ.

i) Acetylation.

Pooled DOC from both control and experimental was reapplied

Figure 7. Oxidation (CrO_3) Followed by Acetylation of Isolated Labelled Corticosterone.

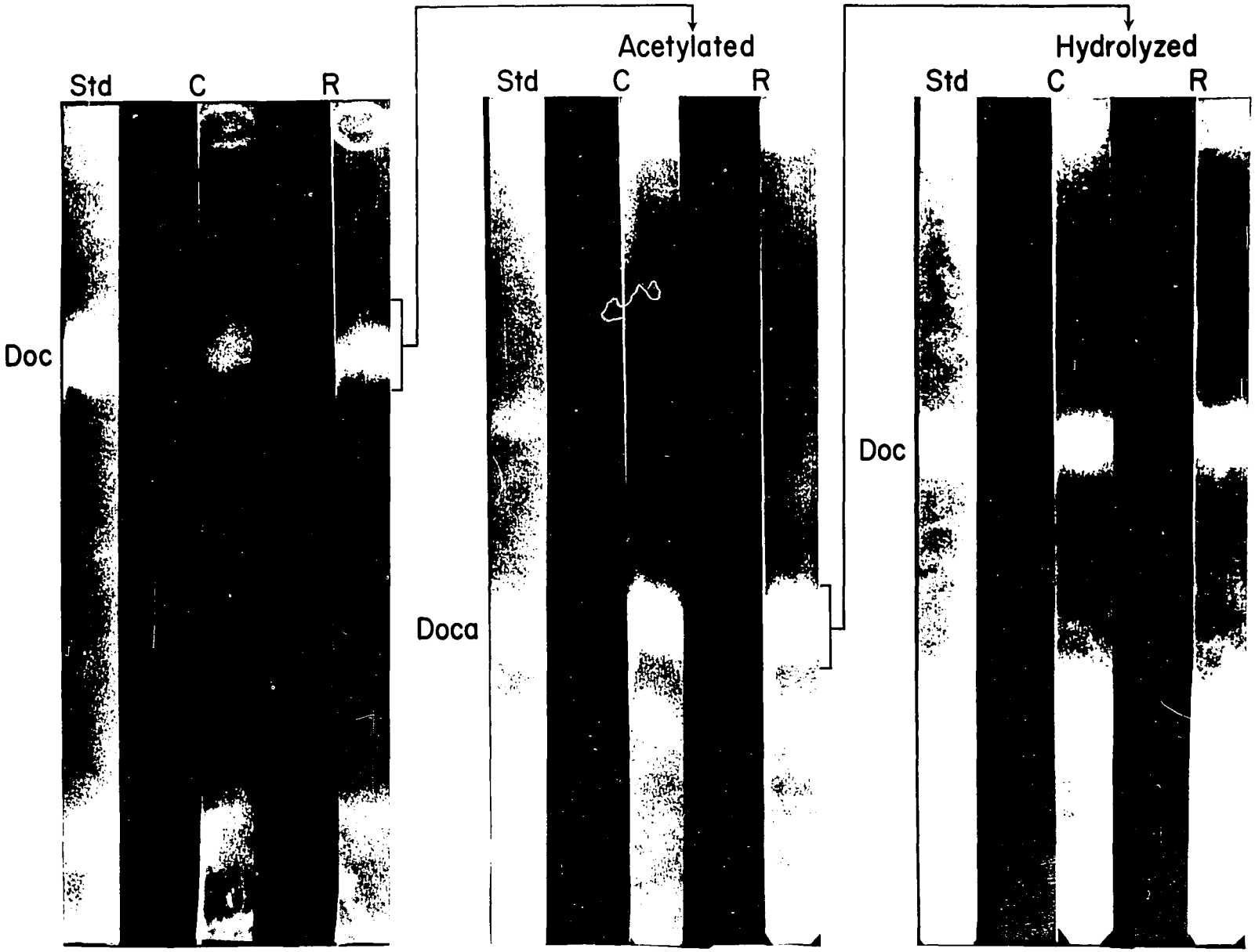
OXIDATION (CrO_3)
FOLLOWED BY ACETYLATION OF ISOLATED LABELLED CORTICOSTERONE



C = Control
R = Reserpinized

Figure 8. Acetylation and Hydrolysis of Isolated Labelled 11-Deoxycorticosterone.

ACETYLATION AND HYDROLYSIS OF ISOLATED LABELLED 11-DEOXYCORTICOSTERONE



C = Control
R = Reserpinized

on the LPG system for 30 hours. Radioactive and ultraviolet-positive zone with similar mobility as DOC was cut, eluted and part of it reapplied on Bush A system as shown on Figure No. 8. ^{14}C-labelled DOC and authentic DOC showed similar chromatographic behaviour after fast acetylation. ^{14}C-labelled deoxycorticosterone 21-Acetate behaved as a single radioactive zone in system D with the same mobility as authentic deoxycorticosterone-21-acetate. This zone on hydrolysis with sodium carbonate yielded free deoxycorticosterone which had the same chromatographic mobility in the D system as the standard.

Section D

"Unknown Compound"

In the beginning, in an experiment with labelled cholesterol a strong radioactive zone in fraction D (Figure No. 1) having similar mobility as 17α-hydroxyprogesterone was found. This zone was positive to ultraviolet light showing a maximum absorption at 240 mμ (characteristic of Δ4-3 keto). It was of particular interest to identify this compound, since it appeared in reserpinized rat adrenal gland homogenates only.

Before starting any rigorous methods of identification, an aliquot of this unknown compound was applied on the

paperchromatogram of Bush B5 system for five hours. Two radioactive zones were found, one having a similar running rate of 18-OH-DOC (more polar or M) and another running with solvent front (less polar or L). The amount of radioactivity distributed was 53% in the more polar and 47% in the less polar. Each of these zones (M and L) on further chromatography on Bush B5 system showed similar splits. Successive chromatography also showed similar splits as shown on Table No. VIII.

The remainder of the unknown compound was applied on Bush B5 system for five hours and the resulting splits subsequently chromatographed on the LPG and Bush B5 system. The results are shown on Table No. IX.

The more polar compound (M) exhibited a positive Porter-Silber reaction but gave a negative blue tetrazolium reaction, as reported by Birmingham for 20 $\rightarrow$ 18 hemiketal of 18-hydroxy-11-deoxycorticosterone. However, the less polar (L) did not react with either method. These results are partially in agreement with the report of Dominguez (30) who obtained a similar metabolite (like our unknown compound) from normal rat adrenal gland homogenates from 21-^{14}C-deoxycorticosterone. It was quite apparent from our results that we were dealing

TABLE VIII

"UNIDENTIFIED COMPOUND"

Percent Distribution of Radioactivity into Products M and L upon Successive Rechromatography.

Starting Compound	1st Rechromat.	2nd Rechromat.	3rd Rechromat.
LPG 30 hours	BUSH B5	BUSH B5	BUSH B5
			M=80
		M=91	
			L=20
	M=53		
			M=32
		L=9	
			L=68
UNKNOWN ^{14}C			
			M=73
		M=40	
			L=27
	L=47		
			M=27
		L=60	
			L=73

TABLE IX

"UNIDENTIFIED COMPOUND"

Percent Distribution of Radioactivity into Products M and L upon Successive Rechromatography.

Starting Compound	1st Rechromat.	2nd Rechromat.	3rd Rechromat.
LPG 30 hours	BUSH B5 5 hours	LPG 30 hours	BUSH B5 5 hours
			M=75
		M=97	
			L=25
	M=80		
			M=26
		L=3	
			L=74
UNKNOWN ^{14}C			
			M=79
		M=49	
			L=21
	L=20		
			M=19
		L=51	
			L=81

with a similar situation as reported by Dominguez.

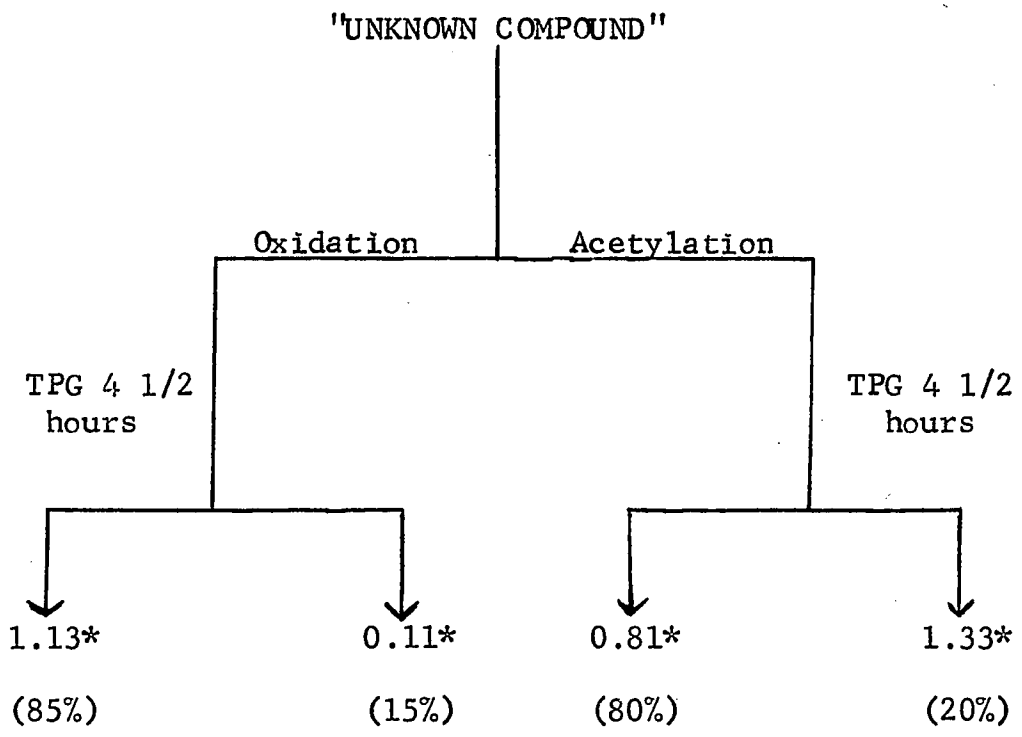
At this point the amount of the unknown compound left was very small. Another similar experiment with cholesterol-4-^{14}C was conducted. This time results showed that not only the unknown compound was present in reserpinized-treated adrenal glands of the rats but also in the control.

This time a part of the compound was taken from both control and experimental and subjected to acetylation and a part to oxidation. The results obtained are summarized on Table No. X. These results are in agreement with Birmingham et al (25) for the identification of 18-OH-DOC.

Still, it seems that certain factors must have been involved in the persisting splitting of the unknown compound. Dominguez suspected that certain solvents, expecially methanol, might be responsible for the production of this unstable unknown compound. According to his report, methanol produces certain changes of authentic 18-OH-DOC, thus resulting into two interconvertible forms, M and L.

To explore the possibility of stability of authentic 18-OH-DOC, five tubes each containing 100 µg of authentic compound were treated as following:

TABLE X



* Rf values with respect to DOC

The figures in parenthesis represent the % radioactivity in each zone.

1. 18-OH-DOC + 10 ml MeOH (dist.)
2. 18-OH-DOC + 10 ml MeOH
   + 0.1 ml H_2O
3. 18-OH-DOC + 10 ml of MeOH
   + 1 ml of H_2O
4. 18-OH-DOC + 10 ml MeOH
   + 0.1 ml HCl 0.1N
5. 18-OH-DOC + 10 ml MeOH
   + 0.1 ml NaOH 0.1N

All tubes were left at room temperature for 24 hours and then dried under nitrogen.

The results are shown on Figure No. 9. It is clear that use of acid produces the biggest change in 18-OH-DOC. However, little, if any changes were observed with methanol.

It seems reasonable to assume that the unknown compound isolated in the present studies is similar to that reported by Dominguez as the less polar form of 18-OH-DOC. The molecular arrangement of 18-OH-DOC responsible for the changes in chromatographic mobility and the conditions leading to the formation of splits upon rechromatography are still not elucidated. Since most authors agree on the lack of 17-α hydroxylase activity in the rat adrenal - either normal (80, 81)

Figure 9. Some Conditions Affecting the Stability of Authentic 18-OH-DOC.

SOME CONDITIONS AFFECTING THE STABILITY OF AUTHENTIC 18-OH-DOC

Bush B_5 5 hrs.

or regenerated (82), and this unknown compound exhibits identical chromatographic behaviour as authentic 17α-hydroxyprogesterone, one may suspect that the steroid isolated from regenerated adrenals of rats with hypertension and tentatively characterized as 17α-hydroxyprogesterone by Brownell et al (79) is identical with this L-form of 18-OH-DOC reported by Dominguez and partially confirmed in the present survey.

IV. DISCUSSION

The present studies with hooded adult male rats agrees with numerous previous observations that the chronic administration of reserpine results in a marked diminution of body weight in various animal species (149-151, 153, 154). Subsequent to the first injection at the dose levels used (0.075 mg/100 g b.w.), rats showed a marked anorexia and a spontaneous restriction of their water intake. Since this was observed throughout the entire experiment (5 days) it seems reasonable to assume that the significant loss of body weight was mainly associated with the extremely low intake of food and water. In the few cases in which the rats developed diarrhoea at the onset of treatment, this might have been a contributing factor to their loss of weight.

Our data also substantiate previous studies by other authors that chronic treatment with reserpine induces a considerable hypertrophy of the adrenal gland (148, 150, 151, 155, 156-161). In our studies, adrenals from reserpine-treated rats invariably appeared enlarged, and their weights were significantly higher than those of the control. Grossly, this enlargement appeared to be mostly limited to the adrenal

cortex. Histological examination also indicated that the cortical parenchyma of the adrenal gland was the more affected by this hypertrophy. These histological studies were carried out at the Institute of Pathology, McGill University, (the author is grateful to Miss E. Angus for the histological studies of the rat adrenal glands) and revealed that the only changes in the adrenal medulla in the glands from reserpine-treated animals was a marked depletion of catecholamine-containing granules. (Stained with haemotoxylin and eosin). Since this is a well-known effect of reserpine, actually, it is its major pharmacological action - and has been abundantly substantiated by many workers (182, 183, 186-188), no histological evidence is given in this thesis. However, it is only being mentioned to convey that the rat adrenal medulla had been highly responsive to the drug treatment.

Regarding the hypertrophy of the adrenal cortex, all evidence seems to indicate that it is mediated through the release of ACTH by the anterior hypophysis (157). It is not altogether clear, however, if this increased release of ACTH is due to a direct effect of reserpine on the hypophysis and/or higher centers in the hypothalamus or it is related to the profound stressing action of reserpine. The specific

affect of this drug, releasing catecholamines into the blood, could be in itself a partial cause of this increased ACTH release (206).

As has already been stated in the review of the literature, the studies on the role of reserpine on various parameters of adrenocortical function (production, release and circulating levels of corticosteroids) have yielded highly conflicting or even opposite results. One of the most recent and satisfactorily controlled studies has been the in vitro investigation by Halkerston et al (158) on corticosteroid biosynthesis from endogenous sources with adrenal bisects obtained from reserpinized rats. Comparing the rate of production of corticosteroids with that of adrenal bisects from control rats, it was shown that it was lower (Blue-tetrazolium reaction) in specimens from reserpinized animals. In the few preliminary observations in this thesis these results could be substantiated by measuring the endogenous corticosteroid production by ultraviolet-light absorption method. Since our main interest was to establish at what level of corticosteroidogenesis this reserpine induced partial inhibition of biosynthesis took place, the bulk of our observations have been carried out with the aid of progesterone-

4-^{14}C and cholesterol-4-^{14}C.

The studies with progesterone-4-^{14}C appear to be inconclusive. The total amounts of substrate utilized by rat adrenal quarters (1-day or 5-day treatment) or homogenates (5-day treatment) prepared from reserpinized animals was of the same order of magnitude as that of their corresponding control preparations. Since the amounts of tissues from the reserpinized animals were higher than in the control, when the values of conversion were calculated on an equal adrenal weight basis (100 mg), it was shown that the utilization of progesterone-4-^{14}C was 10-30% lower in these preparations than in the control. These studies also appeared to indicate that there was a slight selective impairment of 11β-hydroxylase activity in the reserpinized adrenals. However, since the variations between control and experimental were relatively small and the number of observations, under identical <u>in vitro</u> conditions, were limited for statistical analysis, few if any definite conclusions could be derived from the studies.

One incidental finding of certain interest in these studies which corroborate observations with bovine adrenals in this laboratory (207) is that while rat adrenal quarters

incorporated progesterone-4-^{14}C into aldosterone in substantial amounts, the studies with homogenized preparations showed virtually no radioactivity in the chromatographic position of this steroid, in spite of their greater enzymatic activity. This has been attributed to the type of coenzymatic supplementation used in the homogenized preparation (208). However, in the present study, homogenates were also incubated without an exogenous NADPH generating system, and the same lack of substantial incorporation of radioactivity into aldosterone was observed as when NADP plus G-6-P were added.

The studies with cholesterol-4-^{14}C were carried out exclusively with homogenates. No attempts were made with surviving adrenal sections, since previous studies have shown that under standard incubating conditions cortical slices do not metabolize the C_{27} sterol to an appreciable extent (54).

Similar to the studies with progesterone-4-^{14}C, the degree of total utilization of cholesterol-4-^{14}C (2 μc), was found to be comparable in adrenal homogenates from control and reserpinized animals. Homogenates in the former preparation, however, contained considerable less tissue (280-387 mg) than in the latter (347-470 mg). Consequently expressing the activity of the system in terms of equivalent amounts of

tissues, revealed the lower capacity of reserpinized adrenals to utilize cholesterol-4-^{14}C than the controls. The number of comparable observations with cholesterol-4-^{14}C permitted statistical analysis, and the data revealed that the results were significant at the level of 5%. The study of individual conversion products also suggested that reserpinized adrenals incorporated less cholesterol-4-^{14}C into corticosterone. However, statistical analysis revealed that these data were not significant.

It seems, therefore, that the lower production of corticosteroids observed by other authors (158) in studies with adrenal bisects from reserpinized rats is mainly due to some interference of the drug in the cholesterol cleavage mechanism, rather than in the hydroxylations in the progesterone molecule. Although it is generally considered that the endogenous precursor of steroids is cholesterol, this seems a rather surprising finding in view of the fact that the hypertrophy of the adrenal gland resulting from reserpine treatment is due to increased ACTH release (156-158). While there are some disagreements on the subject (209), it is also almost generally considered since Hechter's work (35), that the site of action of ACTH is precisely the enzymatic cleavage of

cholesterol. There might be various possible explanations of this discrepancy between the cellular trophic action of ACTH in reserpinized adrenals and the diminution of the cholesterol cleavage enzymatic activity. However, to discuss them in this thesis would be to speculate without much experimental support. The lack of significant results with reserpine on the hydroxylations of progesterone-4-^{14}C on one hand, and the non-participation of the adrenal medulla on the in vitro utilization of cholesterol-4-^{14}C (54) plus the lack of influence of the catecholamines in the rate of utilization of the C_{27} sterol by cortical homogenates (207) on the other, seem to rule out our working hypothesis that the action of reserpine on corticosteroidogenesis might be associated with a depletion of adrenomedullary enzymes along with pressor amines and medullary nucleotides (205).

As stated before, this working hypothesis was mainly based on the previous findings that the bovine adrenal medulla could hydroxylate in vitro progesterone-4-^{14}C at carbons 11, 17 and 21 (54). The studies also appear to rule out that the deficit in the hydroxylation of progesterone-4-^{14}C by homogenates of enucleated rat adrenals observed by Brownie and Skelton (146) bears a close relationship to the lower production of corticosteroids by reserpinized adrenal rats reported by Halkerston et al (158).

V. SUMMARY

The findings in the present thesis are:

1. That reserpine affects the total body weight of rats as has been reported by other authors in various animal species.
2. That reserpine induced a hypertrophy of the adrenal gland, and that this hypertrophy was mostly due to an increase in cortical tissue.
3. That rat adrenal preparations from reserpine-treated animals produced a slight diminution in total utilization of progesterone-4-^{14}C as well as in incorporation into corticosterone when values were expressed on an equal weight basis. However, no statistical analysis could be carried out to ascertain the significance of these findings.
4. That treatment with reserpine induced a significant diminution in the *in vitro* utilization of cholesterol-4-^{14}C by rat adrenal homogenates.

This is the first instance in which the action of reserpine on corticosteroidogenesis has been explored with the aid of isotopically labelled substrates. The data with cholesterol-4-^{14}C agrees with the diminution of corticosteroid synthesis from endogenous sources brought about by treatment

of reserpine claimed by other authors. The data suggest that the action of reserpine on steroid production is at the biosynthetic station involving the cleavage of cholesterol.

BIBLIOGRAPHY

1. Arnold, J.: Virchow, Arch., 35, 64, 1866.

2. Brown-Sequard, C.E.: C.R. Acad. Sci., 43, 422, 1856.

3. Swingle, W.W., Pfiffner, J.J.: Science, 71, 489, 1930.

4. Kendall, E.C.: Proc. Staff Meet. Mayo Clinic, 9, 245, 1934.

5. Reichstein, T.: Helv. chim. Acta.; 19, 1107, 1936.

6. Wintersteiner, O. and Pfiffner, J.J.: J. Biol. Chem., 116, 291, 1936.

7. Williams, R.H. Editor, Endocrinology, Saunders 1963, ch. 5.

8. Bush, I.E.: J. Physiol., 115, 12P, 1951.

9. Bush, I.E.: J. Endocrinol., 9, 95, 1953.

10. Singer, B. and Stach-Dunne, M.P.: J. Endocrinol., 12, 130, 1955.

11. Péron, F.G.: Endocrinology, 66, 458, 1960.

12. Lucis, O.J., Dyrenfurth, I., and Venning, E.H.: Cand. J. Biochem. physiol., 39, 901, 1961.

13. Péron, F.G.: Endocrinology, 70, 386, 1962.

14. Guillemin, R., Clayton, G.W., Smith, J.D. and Limpscomb, H.S.: Endocrinology, 63, 349, 1958.

15. Eisenstein, A.B. and Strack, I.: Endocrinology, 68, 121, 1961.

16. Ward, P.J. and Birmingham, M.K.: Acta Endocrinol., 39 110, 1962.

17. Lucis, R., Carballeira, A., Venning, E.H.: Steroids, 6, 737, 1965.

18. Giroud, C.J.P., Stachenko, J. and Venning, E.H.: Proc. Soc. Exper. Biol. Med., 92, 154, 1956.

19. Sheppard, H., Swenson, R. and Mowies, T.F.: Endocrinology, 73, 819, 1963.

20. Stachenko, J. and Giroud, C.J.P: Endocrinology, 64, 743, 1959.

21. Stachenko, J. and Giroud, C.J.P.: Endocrinology, 64, 730, 1959.

22. Simpson, S.A.S., Tait, J.F., Wettstein, A., Neher, R., von Euw, J. and Reichstein, R.: Experientia, 9, 333, 1953.

23. Simpson, S.A.S., Tait, J.F. Wettstein, A. Neher, R., von Euw, J., Schlinler, O. and Reichstein, T.: Experientia, 10, 132, 1954.

24. Giroud, C.J.P., Saffran, M., Schally, A.V., Stachenko, J. and Venning, E.H.: Proc. Soc. Exper. Biol. Med., 92, 855, 1956.

25. Birmingham, M.K., Ward, P.J.: J. Biol. Chem., 236, 1661, 1961.

26. Péron, F.G.: Endocrinology, 69, 39, 1961.

27. Reif, A.E. and Longwell, B.B.: Endocrinology, 60, 382, 1956.

28. Hoffman, F.G.: Endocrinology, 60, 382, 1957.

29. Birmingham, M.K.: Nature, 184, 67, 1959.

30. Dominguez, O.V.: Steroids Suppl. II, 29, 1965.

31. Silber, R.H. and Porter, C.C.: Methods of Biochemical Analysis, 4, 139, 1957.

32. Sandor, T., Lanthier, A.: Acta Endocrinol., 42, 355, 1963.

33. Bloch, K.: Rec. Prog. Horm. Res., 6, 111, 1951.

34. Bloch, K.: J. Biol. Chem., 156, 255, 1944.

35. Hechter, O., Zaffaroni, Jacobson, R.P., Levy, H., Jeanloz, R.W., Schenker, V. and Pincus, G.: Rec. Prog. Horm. Res., 6, 215, 1951.

36. Grant, J.K.; Symp. Biochem. Soc., 18, 24, 1960.

37. Grant, J.K.: Lipids Steroid Metabolism "Comparative Biochemistry" III M. Florkin and H.S. Mason (eds.) Academic Press, 1962, p. 163.

38. Wright, L.D.: Annual. Rev. Biochem., 30, 525, 1961.

39. Johnson, J.D. and Bloch, K.: J. Am. Chem. Soc., 79, 1145, 1957.

40. Zaffaroni, A., Hechter, O. and Pincus, G.: Fed. Proc., 10, 150, 1951.

41. Saba, N. and Hechter, O.: Arch. Biochem. Biophys., 51, 457, 1954.

42. Lynn, W.S., Jr., Staple, E. and Gurin: Fed. Proc., 14, 783, 1955.

43. Constantopoulos, G. and Tchen, J.J.: J. Biol. Chem., 236, 65, 1961.

44. Halkerston, I.D.K., Eichhorn, J. and Hechter, O.: J. Biol. Chem., 236, 374, 1961.

45. Solomon, S., Leviton, P. and Lieberman, S.: Rev. Can. Biol., 15, 282, 1956.

46. Shimizu, K., Dorfman, R.I. and Gut, M.: J. Biol. Chem., 235, 25 PC, 1960.

47. Shimizu, K., Hayano, M., Gut, M. and Dorfman, R.I.: J. Biol. Chem., 236, 695, 1961.

48. Shimizu, K., Gut, M. and Dorman, R.I.: J. Biol. Chem., 237, 699, 1962.

49. Constantopoulos, G., Satoh, P.S. and Tchen, T.T.: Biochem. Biophys. Res. Commun, 8, 50, 1962.

50. Samuels, L.T., Helmreich, M.L., Lasater, M.B. and Reich, H.: Science, 113, 490, 1951.

51. Talalay, P. and Wang, V.S.: Biochim.Biophys. Acta, 18, 300, 1955.

52. Aoshima, Y., Kochahian, C.D. and Jadrijevic, D.: Endocrinology, 74, 521, 1964.

53. Samuels, L.T.: Ciba Foundation coll. in Endocrinology, Little Brown & Co., VII, 1953, p. 176.

54. Carballeira, A., Mehdi, A. and Venning,E.H.: Proc. Soc. Exper. Biol. Med., 117, 751, 1965.

55. Byer, K.F. and Samuels, L.T.: J. Biol. Chem., 219, 69, 1956.

56. Kowal, J., Forchielli, E. and Dorfman, R.I.: Steroids, 3, 531, 1964.

57. Kowal, J., Forchielli, E. and Dorfman, R.I.: Steroids, 4, 77, 1964.

58. Baille, A.H., Cameron, E.H.D., Griffiths, K. and Hart, D.M.K.: J. Endocrinol., 31, 227, 1965.

59. Levy, H., Deane, H.W. and Rubin, B.C.: Endocrinology, 65, 932, 1959.

60. Grant, J.K.: Brit. Med. Bull., 18, No. 2, 1962.

61. Dorfman, R.I., Hayano, M., Haynes, R. and Savard, K., Ciba Foundation Coll. in Endocrinology, Little Brown & Co., VII, 1953, p. 191.

62. Sweat, M.L.: J. Am. Chem. Soc., 73, 4056, 1951.

63. Hayano, M. and Dorfman, R.I., J. Biol. Chem., 193, 175, 1951.

64. Grant, J.K.: Biochem. J., 64, 559, 1956.

65. Tomkins, G.M., Michael, P.J. and Curran, J.F.: Biochem. Biophys. Acta, 23, 655, 1957.

66. Sharma, D.C., Forchielli, E. and Dorman, R.I.: J. Biol. Chem., 273, 1495, 1962.

67. Tomkins, G.M., Curran, J.F., Michael, P.J.: Biochim. Biophys. Acta, 28, 449, 1958.

68. Hayano, M., Saba, N., Dorman, R.I. and Hechter, O.: Rect. Prog. Horm. Res., 12, 59, 1956.

69. Cooper, D.Y. and Rosenthal, O.: Arch. Biochem. Biophys., 96, 96, 1962.

70. Cooper, D.Y. and Rosenthal, O.; Arch. Biochem. Biophys., 96, 33, 1962.

71. Lynn, W.S. Jr. and Brown, R.H.: J. Biol. Chem., 232, 1015, 1958.

72. Ryan, K.J. and Engle, L.L.: J. Biol. Chem., 225, 103, 1957.

73. Phychoyos, S., Tallan, H.H. and Grungara, R.: Fed. Proc., 24, 449, 1965.

74. McCarthy, J., Péron, F.G. and Guerra, F.C.: VI Congreso Panamerican De Endocrinologia (Mexico). Excerpta Medica. Intern. Congr. Series No. 99, 1965, abstract 177.

75. Nahamura, Y and Tamoki, B.: Biochim. Biophys. Acta., 85, 150, 1964.

76. Giroud, C.J.P., Stachenko, J. and Venning, E.H.: Proc. Soc. Exper. Biol. Med., 92, 154, 1956.

77. Heard, R.D.H., O'Donnell, R.J.V., Peron, F.G., Saffran, J.C., Solomon, S.S., Thompson, L.M., Willoughby, H., and Yates, C.H.: Prog. Horm. Res., 9, 383, 1954.

78. Eisenstein, A.B.: Proc. Soc. Exper. Biol. Med., 91, 657, 1956.

79. Brownell, K.A., Lee, S.L., Beck, R.R.; Besch, P.K.: Endocrinology, 72, 167, 1963.

80. Birmingham, M.K., Kurlents, E.: Can. J. Biochem. Physiol., 37, 510, 1959.

81. Ward, P.J. and Birmingham, M.K.: Biochem. J., 76, 269, 1960.

82. Laplante, C., Giroud, C.J.P. and Stachenko, J.: Endocrinol., 75, 825, 1964.

83. Hayano, M., Dorfman, R.I.: J. Biol. chem., 201, 175, 1953.

84. Saffran, M. and Bayliss, M.J.: Endocrinology, 52, 140, 1953.

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

86. Hayano, M., Lindberg, M.C., Dorfman, R.I., Hancock, J.E.H., Doering, W. von E.; Arch. Biochem. Biophys., 59, 529, 1955.

87. Bloom, B.M., Hayano, M., Saito, A., Stone, D. and Dorfman, R.I.: Fed. Proc., 15, 222, 1956.

88. Hayano, M., Saito, A., Stone, D. and Dorfman, R.I.: Biochim. Biophys. Acta., 21, 381, 1956.

89. Sweat, M.L. and Lipcomb, M.D.: J. Am. chem. Soc., 177, 5185, 1955.

90. Lynn, W.S. Jr. and Brown, R.H.: J. Biol. chem., 232, 1005, 1958.

91. Haynes, R.C. Jr. and Berthet, L.: J. Biol. chem., 225, 115, 1957.

92. Koritz, S.B., Péron, F.G.: J. Biol. chem., 230, 343, 1958.

93. Koritz, S.B., Péron, F.G.: J. Biol. chem., 234, 3122, 1959.

94. Smith, P.E.: Am. J. Anat., 45, 205, 1930.

95. Ingle, D.J. and Kendall, E.C.: Science, 86, 245, 1937.

96. Ingle, D.J. and Higgins, G.M.: Anat. Rec., 71, 363, 1938.

97. Sayers, G., White, A. and Long, N.N.: J. Biol. Chem., 149, 425, 1943.

98. Li, C.H., Evans, H.M. and Simpson, M.E.: J. Biol. chem., 149, 413, 1943.

99. Goodsell, J.E.: Am.J. Physiol., 134, 119, 1941.

100. Schneppenhein, P. and Hun, A.: Beitr. Path. Anat., 115, 119, 1955.

101. Bergner, G.E., Deane, H.W.: Endocrinology, 43, 240, 1948.

102. Allen, J.M.: Endocrinology, 61, 368, 1957.

103. Race, G.J., Nickey, W.M., Wolf, P.S., Jordan, E.J.: A.M.A. Arch. Path., 64, 312, 1957.

104. Lipcomb, H.S. and Nelson, D.H.: Endocrinology, 66, 144, 1960.

105. Guilleman, R., Glayton, G.W., Smith, J.P. and Lipcomb, H.S.: Endocrinology, 63, 349, 1958.

106. Wettstein, A.: Experientia, 17, 329, 1961.

107. Haynes, R.C. Jr.: J. Biol. Chem., 233, 1220, 1958.

108. Haynes, R.C. Jr., Koritz, S.B. and Péron, F.G.: J. Biol. Chem., 234, 1421, 1959.

109. Péron, F.G.: J. Biol. Chem., 236, 1764, 1961.

110. Selye, H., Stone, H.: "On the experimental morphology of the adrenal cortex", Thomas Springfield, 1950.

111. Heard, R.D.H. and Raper, H.S.: Biochem. J., 27, 36, 1933.

112. Gurin, S. and Delluva, A.M.: J. Biol. chem., 170, 545, 1947.

113. Goodal, McC and Kirshner, N.: J. Biol. chem., 226, 213, 1957.

114. Kaufman, S.: J. Biol. chem., 226, 511, 1957.

115. Kaufman, S.: J. Biol. chem., 234, 2677, 1959.

116. Kaufman, S., J. Biol. chem., 236, 804, 1961.

117. Kaufman, S. and Levenberg, B.: J. Biol. chem., 234, 2683, 1959.

118. Fellman, J.H. and Delin, M.K.: Biochim. Biophys, Acta, 28, 328, 1958.

119. Rosenfeld, G., Leeper, L.C. and Underfriend, S.: Arch. Biochem. Biophys., 74, 252, 1958.

120. Boylen, J.B. and Ovastel, J.H.: Biochem. J., 80, 644, 1961.

121. Weiner, N.: "The Hormones" p. 403 G. Pincus, Academic Press IV, 1964.

122. Langemann, H.: Brit. J. Pharmacol., 6, 318, 1951.

123. Blaschho, H.: Pharmacol. Revs., 6, 23, 1954.

124. Green, D.E., Leloir, L.F. and Nocito, V.: J. Biol. chem., 161, 559, 1945.

125. Hagen, P.: J. Pharmacol. Exptl. Therap., 116, 26, 1956.

126. Hagen, P. and Welch, A.D.: Rec. Prog. Horm. Res., 12, 27, 1955.

127. Kirshner, N.: J. Biol. Chem., 226, 821, 1957.

128. Levin, E.Y., Levenberg, B. and Kaufman, S.: J. Biol. Chem. 235, 2080, 1960.

129. Bulbring, E.: Brit. J. Pharmacol., 4, 234, 1949.

130. Kirshner, N.: Pharmacol. Revs., 11, 350, 1959.

131. Kirshner, N., Goodall, McC.: Biochim. Biophys. Acta, 24, 658, 1957.

132. Cantoni, G.L.: J. Biol. Chem., 204, 403, 1953.

133. Axelrod, J.: Fed. Proc., 20, 236, 1961.

134. Holzbauer, M. and Vogt, M.: J. Physiol., 138, 449, 1957.

135. Giroud, C.J.P.: In Muller, A.F. and O'Corner, C.M., (eds.): An International Symposium on Aldosterone, J.A. Churchill Ltd., London, p. 99, 1958.

136. Fortier, C. and De Groot, J.: Amer. J. Physiol., 196, 589, 1959.

137. Bernstein, D.E., Biskind, G.R. and Zenker, N.: Program. 40th Meeting Endocrine Soziety, June 1958, p. 13.

138. Kolthoff, N.J., Macchi, I.A. and Wyman, L.C.: Endocrinology, 73, 27, 1963.

139. Koch, B., Miahepvoloss, C. and Stutinsky, F.: C.R. Acad. Sci., 254, 744, 1962.

140. Koch, B., Miahepvoloss, C. and Stutinsky, F.: C.R. Acad. Sci., 254, 3436, 1962.

141. Brogi, M.P. and Pellegrino, C.: J. Physiol., 146, 165, 1959.

142. Masson, G.N.C., Koritz, S.B. and Peron, F.G.: Endocrinology, 62, 229, 1958.

143. Macchi, I.A., Wyman, L.C.: Endocrinology, 73, 20, 1963.

144. Hyde, P.M. and Skelton, F.R.: Endocrinology, 69, 250, 1961.

145. Birmingham, M.K., Rochefort, G. and Traikov, H.: Endocrinology, 76, 819, 1965.

146. Brownie, A.C. and Skelton, F.R.: Steroids, 6, 47, 1965.

147. Mueller, J.M., Schlittler, E. and Bein, H.G.: Experientia, 8, 338, 1952.

148. Gaunt, R., Renzi, A.A., Antonchak, N., Millar, G.J. and Gilman, M.: Ann. N.Y. Acad. Sci., 59, 22, 1954.

149. Eränkö, O., Hopsu, V., Kivalo, E. and Telkkä, A.: Nature, 180, 1130, 1957.

150. Hertting, G. and Hornykicwicz, O.: Acta. Endocrinol., 26, 204, 1957.

151. Sackler, A.M., Weltman, A.M., Bradshaw, M. and Heilman, F.: Acta. Endocrinol., 34, 619, 1960.

152. Giuliani, G., Motta, M. and Martini, L.: Acta. Endocrinol., 51, 203, 1966.

153. Winthrington, P. and Zaimis, E.: Brit. J. Pharmacol., 17, 380, 1961.

154. Marhicwicz, L.: Acta. Physiol. Scand., 58, 376, 1963.

155. Epstein, R., Erikson, L.B. and Reynolds, S.R.M.: Endocrinology, 66, 167, 1960.

156. Khazan, N., Sulman, F.G. and Winnik, H.Z.: Proc. Soc. Exper. Biol. Med., 106, 579, 1961.

157. Kitay, J.I., Holub, D.A. and Jailer, J.W.: Endocrinology, 65, 548, 1959.

158. Halkerston, I.D.K., Feinstein, M. and Hechter, O.: Proc. Soc. Exper. Biol. Med., 115, 292, 1964.

159. Monti, M., Benedetti, N., Reissenweber, N.J., Appetauer, L.C., Dominguez, J. and Grino, E.: Arch. Int. Pharmacody, 155, 236, 1965.

160. Chatterjee, A.: Endocrinologie, 48, 265, 1965.

161. Denti, A., Nallar, R. and Lantos, C.P.: Acta. Physiol. Lat. Amer., 13, 324, 1963.

162. Egdahl, R.H., Richards, J.B. and Hume, D.N.: Science, 123, 418, 1956.

163. Harwood, C.H. and Mason, J.W.: Endocrinology, 60, 239, 1957.

164. Newcomer, W.S.: Am. J. Physiol., 202, 337, 1962.

165. Eechaute, W.: Arch. Int. Pharmacody., 139, 403, 1962.

166. Maickel, R.P., Westermann, E.O. and Brodie, B.B.: J. Pharmacol. Exp. Ther., 134, 167, 1961.

167. Cushman, P. and Hilton, J.G.: J. Endocr., 31, 181, 1965.

168. Westerman, E.O., Maickel, R.P. and Brodie, B.B.: J. Pharmacol. Exp. Ther., 138, 206, 1962.

169. Rosenthal, N.R.: Psychophermacologia, 2, 55, 1961.

170. Saffran, M. and Vogt, M.: Brit. J. Pharmacol., 15, 165, 1960.

171. Bertles, A., Carlsson, A. and Rosengren, E.: Naturwissenschaften, 43, 521, 1956.

172. Burach, W.R., Weiner, N. and Hagen, P.: J. Pharmacol. exptl. therap., 130, 245, 1961.

173. Burn, J.H., Philpot., F.J. and Trendelinburg, U.: Brit. J. Pharmacol., 9, 423, 1954.

174. Carlsson, A. and Hillarp, N.A.: Fysiograf. Sällskap. Lund, Forch., 26, 90, 1956.

175. Carlsson, A., Rosengren, E., Bertler, A. and Nilsson, J.: In "Psychotropic Drugs", p. 363, E. Garratini, S. Elsevier, Amsterdam, Holland, 1957.

176. Holzbauer, M. and Vogt, M.: J. Neurochem., 1, 8, 1956.

177. Paasonen, M.K. and Krayer, O.: J. Pharmacol. Exptl. therap., 123, 153, 1958.

178. Wood, D.R., Kollegoda, S.R. and Krayer, O.: J. Pharmacol. exptl. therap., 124, 340, 1958.

179. Holtz, P., Balzer, H., Westermann, E.: Arch. exptl. pathol. pharmakol. Naunyn-Schmiedebergs, 231, 361, 1957.

180. Coupland, R.E.: Nature, 181, 930, 1957.

181. Brodie, B.B., Olin, J.S., Kuntzman, R.E. and Shore, P.A.; Science, 125, 1293, 1957.

182. Koreberg, G. and Schuman, H.J.: Arch. exptl. pathol. pharmakol. Naunyn-Schmiedebergs, 231, 349, 1957.

183. Koreberg, G. and Schuman, H.J.: Arch. exptl. patjol. pharmakol. Naunyn-Schmiedebergs, 232, 278, 1957.

184. Koreberg, G. and Schuman, H.J.: Arch. exptl. pathol. pharmakol. Naunyn-Schmiedebergs, 234, 133, 1958.

185. Stjärne, L. and Shapiro, S.: Nature, 184, 2023, 1959.

186. Callingham, B.A. and Mann, M.: Nature, 181, 423, 1958.

187. Callingham, B.A. and Mann, M.: Nature, 182, 1020, 1958.

188. Mirkin, B.L.: Nature, 182, 113, 1958.

189. Umbreit, W.W., Burris, R.H. and Stauffer, J.F., "Manometric Techniques". Burgen Publishing Co., Minneapolis, 1957, p. 149.

189a). Carballeira, A. and Venning, E.H.: Steroids, 4, 329, 1964.

190. Bush, I.E.:Biochemical J., 50, 370, 1952.

191. Zaffaroni, A. and Burton, R.B.: J. Biol. Chem., 193, 749, 1951.

192. Ulick, S. and Vetter, K.K.: J. Biol. Chem., 237, 3364, 1962.

193. Eberlein, W.R. and Bongiovanni, A.M.; Arch. Biochem., 59, 90, 1955.

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

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

196. Bush, I.E.: The Chromatography of Steroids, Pergamon Press, 1961, p.358.

197. Bush, I.E.: The Chromatography of Steroids, Pergamon Press, 1961, p.360.

198. Bush, I.E.: The Chromatography of Steroids, Pergamon Press, 1961, p.363.

199. Nowaczynski, W., Goldner, M. and Genest, J.: J. Lab. Clin. Med., 45, 818, 1955.

200. Porter, C.C. and Silber, R.H.: J. Biol. Chem., 185, 201, 1950.

201. Lewbart, M.L. and Mattox, V.R.: Fed. Proc., 19, 158, 1960.

202. Tsang, C.P.W.: Msc thesis, McGill University, 1965.

203. Schneider, J.A.: in "Rauwolfia" Woodson, R.E., Youngken, H.W., Schlittler, E. and Schneider, J.A. (Eds.), Little, Brown and Co., 1957, p. 50.

204. Wurtman, R.J. and Axelrod, J.: Science, 150, 1465, 1965.

205. Burack, W.R., Weiner, N. and Hagen, P.B.: J. Pharmacol. Exptl. Therp., 130, 245, 1960.

206. Long, C.N.H. and Fry, E.M.: Proc. Soc. Exper. Biol. Med., 59, 67, 1945.

207. Carballeira, A.; PhD thesis, McGill University, 1966.

208. Kahnt, F.W. and Neher, R.: Helv. Chim. Acta., 48, 1457, 1965.

209. Karaboyas, G.C. and Koritz, S.B.: Biochemistry, 4, 462, 1965.