

OXIDATION AND REDUCTION OF THE C-11 POSITION OF CORTICOSTERONE
AND 11-DEHYDROCORTICOSTERONE BY MOUSE LIVER
FRACTIONS IN VITRO

by

ELIZABETH CAIRINE LEWIS

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in the Department
of
BIOCHEMISTRY

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April, 1962

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Biochemistry

The University of British Columbia,
Vancouver 8, Canada.

Date April 27, 1967

A B S T R A C T

OXIDATION AND REDUCTION OF THE C-11 POSITION OF CORTICOSTERONE
AND 11-DEHYDROCORTICOSTERONE BY MOUSE LIVER

FRACTIONS IN VITRO

Corticosterone and 20 α dihydrocorticosterone have been found in the liver and blood of mice injected intravenously with C-11-dehydrocorticosterone. Within a few minutes after injection the latter is no longer detectable in the blood and liver. (1).

The enzyme responsible for the reduction of the C-11 keto group has been isolated in the microsomal fraction of mouse liver homogenate. This fraction was precipitated by ultracentrifugation at 100,000 x G of the supernatant after centrifugation at 6,000 x G.

NADPH₂ was established as the coenzyme required although limited activity could be demonstrated with NADH₂. The reaction can be reversed by use of NADP.

Certain kinetic properties of the reaction have been investigated. The reaction velocity is proportional to enzyme concentration and linear with time over the period studied. For the reduction reaction with 11-dehydrocorticosterone as substrate $K_m = 1.8 \times 10^{-5}$ and $V_{max} = 1.6 \times 10^{-4}$ μ moles/ml/min. For the dehydrogenation reaction with corticosterone as substrate $K_m = 1.7 \times 10^{-4}$ and $V_{max} = 2.7 \times 10^{-3}$ μ moles/ml/min.

A C K N O W L E D G M E N T S

The author wishes to express her sincere thanks to Dr. Marvin Darrach for his advice and encouragement.

The assistance of the National Research Council in the form of a Studentship is gratefully acknowledged.

TABLE OF CONTENTS

	Page
Title	i
Abstract	ii
Acknowledgments	iii
Table of Contents	iv
Introduction	1
Experimental	11
Results	13
Isolation of 11 β reductase activity	13
Reversibility of the 11 β reductase system	14
Substrate specificity of fraction P ₃	14
Coenzyme dependence of crude 11 β reductase of mouse liver	14
Kinetics of the 11 β reductase system	16
Discussion	26
Summary	31
Bibliography	32

LIST OF TABLES AND FIGURES

	Page
Table I - 11-Dehydrocorticosterone-11 β -Reductase Activity in Mouse Liver Fractions.	15
Table II - Reduction and Dehydrogenation at C-11 of Various Corticosteroids by P ₃ Fraction of Mouse Liver.	17
Fig. I - Effect of Enzyme Concentration on Reaction Velocity (Substrate 11-Dehydrocorticosterone).	20
Fig. II - Effect of Time on the Yield of Corticosterone.	21
Fig. III - Effect of Substrate Concentration on Reaction Velocity (Substrate 11-Dehydrocorticosterone).	22
Fig. IV - Effect of Enzyme Concentration on Reaction Velocity (Substrate Corticosterone)	23
Fig. V - Effect of Time on the Yield of 11-Dehydrocorticosterone.	24
Fig. VI - Effect of Substrate Concentration on Reaction Velocity (Substrate Corticosterone)	25

I N T R O D U C T I O N

The high hepatic concentration of exogenously administered hormones first suggested the importance of the liver in steroid metabolism, including their activation, degradation, and excretion. Metabolism by the liver of the adrenocortical hormones has been demonstrated both in vivo and in vitro. The results have revealed that C-21 steroids are altered by oxidation or reduction at C-11, reduction of the side chain, and reduction of the Δ^4 -3 Keto system of ring A. The 17-keto derivatives have been found as corticosteroid metabolites but these are not major end products. (2).

The demonstration that cortisone is converted to cortisol in vivo first suggested reduction of the C-11 ketone as responsible for some of the activity previously attributed to cortisone. (3). Hechter pointed out that although cortisone is rapidly destroyed by the liver, metabolic activity following administration of the hormone persists for some time. He, therefore, postulated that certain metabolites of cortisone formed in the liver are physiologically active. (4). Ingle observed that cortisone was unable to exert its usual effect on carbohydrate tolerance in the hepatectomized rat, although administration of an adrenocortical extract produced the normal effect of a decrease in glucose tolerance. It seemed indicated, therefore, that cortisone was altered by passage through the liver and the accompanying

changes in the molecule must be necessary before it can affect carbohydrate metabolism. (5). All these observations substantiated the idea that some of the metabolic activity previously attributed to cortisone may in fact be dependent on its conversion to cortisol.

The original biological findings with respect to the exceptionally large difference between the activities of 2-methylcortisone and 2-methylcortisol support the C-11 reduction hypothesis, the low activity possessed by 2-methylcortisone being due to the small amount of 2-methylcortisol which is formed from it. (6). The C-11 ketonic form has been demonstrated to be less active than the corresponding 11 β hydroxy form for several pairs of steroids. It is claimed therefore that biological activity of the 11-oxygenated adrenal steroids is confined to the 11 β hydroxy forms except when the C-11 ketones can be reduced to the former.

The fact that tissues vary in ability to effect reduction of the C-11 keto position was demonstrated when the antirheumatic effect of cortisol could be observed on direct injection into the joint space while cortisone was inactive. (7). This is in keeping with the previous ideas on a liver metabolite as the active form since if cortisone is injected into the circulation it is fully active. Therefore, it seems that there is a discrepancy between the in vivo

findings that cortisone and corticosterone are active over periods of time and the in vivo and in vitro observations that C-11 keto compounds are rapidly metabolized by the liver to a variety of products. The best explanation seems to be that some of the products formed in liver metabolism possess the anti-arthritic, thymolytic, and other biological activities associated with cortisone administration.

Hechter, Frank, Caspi and Frank (8) as well as Schneider and Horstman (9) have demonstrated cortisol to be metabolized by the liver as rapidly as cortisone. However, some binding of the active form to a tissue constituent could explain the prolonged activity despite the rapidity of hepatic alterations in the molecule. Axelrod and Miller postulated that in order for cortisone or cortisol to exert their action, the active form of the hormone must be bound to non-hepatic tissue in order to prevent destruction by the liver through subsequent metabolic changes. (3). Binding of steroid to plasma protein, particularly to the globulin fraction in human plasma has been shown. (10). The active corticosteroid hormones, cortisol and corticosterone, circulate in the plasma largely as a complex formed with an α globulin called the corticosteroid binding globulin. At concentrations where the binding sites of this protein are saturated, binding by albumin and other plasma proteins is observed. (11).

It is now well established that the subsequent reductions of ring A and the side chain of the molecule are responsible for the elimination of biological activity. (12).

In fact, all postulated metabolic schemes show reduction to be the major inactivating mechanism for adrenal cortical steroids in the liver and the chief end products for such metabolism to be saturated polyhydroxylated C-21 steroids. (2,13,14).

It was postulated by Hubener, Fukushima and Gallagher that the reduction of the C-11 keto position of 11-dehydrocorticosterone or cortisone to the 11 β hydroxy form occurred only in compounds having the Δ^4 -3keto ring structure. (15). They were unable to find any reduction in compounds having a saturated ring A. Since this reduction occurred only in Δ^4 -3-keto steroids this was postulated as a possible site of enzyme substrate attachment. This specificity of reduction was questioned by Bush and Mahesh (16) after their demonstration of the occurrence of three 3 α ,11 β -hydroxy-5 α H steroids in the liver but not their corresponding 11-oxo derivatives suggesting that the ring A saturated compounds may have undergone reduction of their C-11 ketone grouping. They presented the hypothesis that the pattern of 11-oxo and 11 β hydroxy steroids found in urine might be explained if the well known oxidation reduction of 11-oxygen functions in vivo was limited to those steroids having a more or less flat α surface over the A and B rings. This appeared reasonable since reduction at C-11 is stereospecific and must presumably occur by association of the α side of the steroid with the enzyme responsible for the reaction. Steroids with a buckled

or bent A/B ring junction would not be likely to combine as firmly with such an enzyme which was already known to act rapidly on the Δ^4 -3-keto forms which have a roughly planar A/B ring system. It was further suggested, therefore, that the substrate specificity of the 11 β -hydroxy-dehydrogenase system was determined largely by the steric properties of the steroid, particularly the A/B ring junction. With one exception occurring in the dog (17), reduction appears to be entirely to the 11 β -hydroxy form and the 11 α -hydroxy epimers of compounds such as cortisol have been shown to be biologically inactive. (12).

Since it appears likely that the adrenal 11-oxosteroids are not themselves biologically active on the tissues affected by adrenocortical hormones but are active when administered systemically only because of their rapid and complete reduction to the 11 β alcohol further studies were carried on and two possibilities were suggested.

The first theory which was supported by Talalay and William-Ashman (18) was that these hormones exerted their action in a manner similar to the coenzymes: in this case by acting as an essential cofactor in a redox system. On the basis of experiments with estradiol and placental isocitric dehydrogenase (19) they proposed that since most steroid dehydrogenases in animal tissue can use both NAD and NADP as coenzyme, steroid hormones in general may exert their action by catalyzing transhydrogenation from reduced NAD to NADP.

They have shown that this catalysis of transhydrogenation is evidently the basis of the stimulation of placental isocitric dehydrogenase in vitro by estradiol that was observed by Vिलlee, and point out that the same dual requirement for pyridine nucleotide exists for the steroid 11 β -hydroxydehydrogenase of rat liver.

Other results obtained by Bush and Mahesh (16), Glenn, Stafford (20), and others favour an alternate general theory: namely, that the 11 β -hydroxy steroid hormones, not the related 11-ketones, are the active form and that oxidation reduction of the C-11 oxygen function is not directly concerned in their mode of action. Bush and Mahesh relate the latter theory to their observations that C-11 reduction occurs in vivo only in steroids having the relatively flat α surface over rings A and B. Thus little, if any, reduction of the 11-oxo steroids occurred with those which have the buckled A/B ring junction typical of 5 β H steroids (11-oxo-aetiocholanone or tetrahydrocortisone). On the other hand, 5 α epimers with flat A/B ring junctions were extensively reduced (11-oxoandrosterone and allotetrahydrocortisone).

Metabolism of corticosteroids has been examined in other tissues. Studies by Sweät and Bryson (21) have shown that cortisol can be degraded in muscle tissue by at least five separate pathways, namely cleavage of the C-17 side chain, oxidation of the C-11 hydroxyl, deoxygenation at C-17 and reduction at C-4,5 and C-20. If the action of cortisol is via

a chemical process it is possible one or more of these reactions is involved in a key hormonal activation process. As cortisol is known to modify a number of physiological processes (electrolytic, glycogenic, anti-inflammatory, etc.) it is also possible that the different chemical conversions are associated with separate physiological processes. The work of Sweat and Bryson confirms the hypothesis that reduction of cortisone to cortisol, believed to be the activation, is primarily a hepatic function (21). In muscle there does not seem to be this marked tendency towards C-11 reduction compared to other reactions. These observations are all consistent with the recognition that cortisone administered systematically has effects comparable with cortisol but when employed locally is much less active. For example, in local anti-inflammatory reactions cortisol has been estimated 7-8 times as effective as cortisone. (22).

With the observation of this characteristic reduction at C-11 in liver oxysteroid metabolism, studies were instituted on the distribution of enzyme systems responsible for the reactions at C-11 of the corticosteroids with respect to their localization in the different tissues and subcellular fractions. Hurlock and Talalay (23) reported the localization in mammalian liver microsomes of an 11β -hydroxy steroid dehydrogenase which catalyzes a freely reversible interconversion of cortisol and cortisone. This enzyme functions equally well with either NAD or NADP as hydrogen carrier. The same workers

also determined the intracellular location of the 11β -hydroxy-dehydrogenase in rat liver by differential centrifugation. The mitochondria and microsome free supernatant were inactive. However, if the microsomes were supplemented with a generating system for reduced NAD, C-11 reduction occurred. Addition of the oxidized coenzyme resulted in the reverse reaction.

Other than liver, kidney has been considered to be the major organ responsible for cortisol and cortisone metabolism. In a study by Mahesh and Ulrich (24) cortisone and cortisol were metabolized either not at all or only to a small extent in the brain, diaphragm, heart, gastrointestinal tract, thymus and skeletal muscle of the rat as compared to the kidney. In particular, oxidation at C-11 occurred to an appreciable extent only in the kidney and appeared to be the main metabolic transformation of cortisol by rat kidney. Reduction at C-11 in cortisone was much less than the oxidation of the reduced compound indicating this enzyme equilibrium is different from the 11β -hydroxy-dehydrogenase found in rat liver. The kidney enzyme, like the liver enzyme, could utilize either NAD or NADP. In the investigation of subcellular fractions to determine the location of the enzyme system, the nuclear and microsomal fractions of kidney homogenate were observed to have the greatest effect on C-11 metabolism. The observation that most of the enzyme activity in rat kidney was associated with microsomes and nuclei would appear to correlate with morphological studies of the two fractions. From electron microscopic studies of liver and

pancreatic microsomes Palade and Seikewitz (25) suggested these particles are derived from the endoplasmic reticulum. Since the membrane of the endoplasmic reticulum is continuous, at least intermittently, with the cell membrane and nuclear membranes this can be postulated as an explanation for the dual location of enzyme activity associated here with metabolism of cortisol and cortisone. In a preparation of isolated nuclei by Dounce's method no oxidation or reduction at C-11 could be demonstrated. This indicated that the activity previously observed associated with a preparation of nuclei contaminated with microsomes must be localized in the microsomal portion. (44).

In an in vivo study on the metabolism of corticosterone and 11-dehydrocorticosterone by the mouse it was observed by Darrach et al (26) that intravenous injection of 11-dehydrocorticosterone resulted in its rapid disappearance and the related appearance of corticosterone. The decay curve for corticosterone in liver was essentially the same as found previously following administration of corticosterone. The metabolite, 20 α -dihydrocorticosterone also accumulates. No traces of C-20 reduced 11-dehydrocorticosterone could be found in blood or liver after any of the time intervals studied. Although the 11-keto form is attacked more readily by this C-20 reductase in vitro it is apparent that in vivo reduction occurs very rapidly at the C-11 ketone and probably prior to reduction of the C-20 group.

At present the 11 β -hydroxyl is recognized as a major

functional group of adrenal corticosteroids (12) and alterations at C-11 represent important metabolic changes. The interconversion of the 11 β -hydroxy and 11-keto forms in vivo has been observed in numerous studies based on analysis of urinary metabolites (27). This reaction has also been demonstrated in perfused liver of rat (23) and dog (17), and in vitro in rat, beef, and pig liver (28), rat kidney (29), bovine muscle (21), and in fibroblasts (30), lymphocytes and other tissues of several species. The liver is probably the main site of reduction of 11-keto steroids to the 11 β -hydroxyl derivatives thus yielding biologically active glucocorticoids. In extrahepatic tissues, however, conditions favour dehydrogenation of the 11 β -hydroxyl to an 11-ketone group (31). Partially purified enzymes have been prepared from microsomes of rat liver (23), kidney (24), and human liver and placenta (32) which catalyze the reversible reduction and dehydrogenation of the C-11 oxygen of the various corticosteroids and which utilize pyridine nucleotides as cofactors. In this study the author wishes to present studies on the isolation and characterization of the mouse liver enzyme responsible for the in vivo observation (1) that the first step in the metabolism by the mouse of intravenously injected 11-dehydrocorticosterone is reduction at the C-11 position to the 11 β -hydroxyl function- the biologically active form (12).

EXPERIMENTAL

Preparation of mouse liver fractions.

One part fresh mouse liver was homogenized with 4 parts of cold 0.25 M sucrose in a glass tissue grinder fitted with a teflon pestle having .006 - .009 inch clearance. The precipitate after centrifugation for 20 minutes at 600 G in an International refrigerated centrifuge was designated P_1 and the supernatant solution S_1 . Fractions P_2 and S_2 were obtained from S_1 after centrifugation for 15 minutes at 6,000 G in a Servall high speed centrifuge and fractions P_3 and S_3 from S_2 after centrifugation for 60 minutes at 100,000 G in the Spinco Model E ultracentrifuge. (34). One part Krebs Ringer phosphate buffer (33) and 5 parts of 0.25 M sucrose were added to each precipitate to bring the volume up to that before centrifugation. Since no loss of enzyme activity occurred on storage at -10°C of frozen P_3 preparations large batches were made at one time, frozen in 25 ml quantities and thawed as required for each experiment.

Incubation and extraction of mouse liver fractions.

The incubation medium, unless indicated otherwise, contained 2 mls of a tissue preparation prepared as outlined above; 65 mg niacinamide, 15 mg sodium fumarate and 3 mg. of nicotinamide-adenine-dinucleotide phosphate, reduced form (NADPH_2), or 4 mg. of the oxidized form (NADP), when the

oxidative reaction was being studied, each added in 2 ml Krebs Ringer phosphate buffer at pH 7.4. (35). The steroid substrate was added in 0.2 ml ethanol and the total volume adjusted to 10 ml. The mixtures were incubated under N_2 at $38^\circ C$ for 2 hours in a Dubnoff incubator shaking at 50 cycles per minute. The incubation mixture was extracted with 5 x 25 ml chloroform which was washed with 10 mls alkali, 10 mls acid and 10 mls water, dried over anhydrous sodium sulphate, filtered through sintered glass and evaporated in vacuo at $40^\circ C$.

Chromatography and Isolation of Compounds.

The residue after evaporation was chromatographed on methanol washed (5X) Whatman No. 1 paper in a benzene formamide system. (36). Chromatograms were run 7-8 hours to give good separation of corticosterone and 11-dehydrocorticosterone, the latter moving approximately twice as far in that time.

The zones were detected by ultra violet contact photographs, the spots cut out and steroid eluted with methanol-chloroform (1:1). Eluates were filtered through sintered glass and dried under N_2 . Isolated compounds were then measured quantitatively by the blue tetrazolium method. (37). This colorimetric determination is specific for steroids with an intact α -ketol side chain. The reducing properties of this side chain are responsible for production of the coloured

formazan complex which gives rise to a specific absorption peak at 520 m μ .

Pooled samples of the products in each case were also characterized by mixed chromatograms with authentic compounds. Sulphuric acid chromogens (38) were obtained from collected samples and the absorption spectra compared with authentic standards.

R E S U L T S

Isolation of 11 β reductase activity.

The present study was first directed towards localization in a fraction of homogenized mouse liver of the 11 β reductase activity. Table 1 records the activity of various fractions of mouse liver in reducing 11-dehydrocorticosterone to corticosterone. Activity was found consistently to be absent from the supernatant following high speed centrifugation and was recovered in the precipitate P₃ to the extent of approximately half that found in whole homogenate (WH) or supernatants after centrifugation at lower speeds. Washing and recentrifugation did not alter the activity. Faint traces of presumably C-20 reduced products were observed on the chromatograms but in amounts too small to characterize. When chromatograms were stained with blue tetrazolium no evidence of ring A reduced metabolites could be observed in the P₃ fraction. In the supernatant fraction both C-20 reduced and ring A reduced metabolites were obtained although a five fold increase in coenzyme concentration was required to demonstrate the latter. In P₃, even with the higher coenzyme levels, no ring A reduction was demonstrable. The substrate and product could be recovered from chromatograms quantitatively within experimental error. For example, in an incubation of 250 μ g of steroid approximately 75-85% could be recovered in product and substrate. This agrees well with the recovery values reported in the literature. (39).

Hence it appears that fraction P_3 does not contain significant amounts of other enzymes attacking either corticosterone or 11-dehydrocorticosterone.

Reversibility of the 11 β reductase system: 11 β hydroxydehydrogenase activity of P_3

It was shown (Table 11) that when the P_3 preparation was incubated with NADP as coenzyme and fumarate omitted, dehydrogenation of corticosterone to 11-dehydrocorticosterone occurred, and in much greater yield than the reduction reaction.

Substrate specificity of fraction P_3 :

As shown in Table 11 the enzyme preparation is capable of effecting the reduction of several substrates: cortisone, 11-dehydrocorticosterone, and 20 α dihydro-11-dehydrocorticosterone. The dehydrogenation of cortisol and corticosterone could also be demonstrated with the P_3 enzyme.

Coenzyme dependence of crude 11 β reductase of mouse liver:

No measurable amount of 11 β reductase activity in fraction P_3 was observed in the absence of added coenzyme. While addition of NAD or NADH₂ resulted in production of a small amount of product the yield was 8 times greater with NADP or NADPH₂ indicating a greater degree of specificity

TABLE I

11-DEHYDROCORTICOSTERONE-11 β -REDUCTASE ACTIVITY
IN MOUSE LIVER FRACTIONS

FRACTION	CORTICOSTERONE FORMED, μ g MEAN
WH	122
S ₁	160
S ₂	138
S ₃	0
P ₃	71

Conditions: 5 ml tissue preparation + 15 mg sodium fumarate + 65 mg niacinamide + 2 mg NADH₂ + 2 mg NADPH₂ in a total of 6 ml Krebs-Ringer Phosphate buffer, pH 7.4; 1 mg 11-dehydrocorticosterone in 0.2 ml ethanol added; incubated under N₂ at 38°C for 2 hours. Neutral chloroform extract of incubation mixture was chromatographed, measured spectrophotometrically, by the U.V. and B-T methods and characterized.

for this coenzyme in both the oxidizing and reducing reactions. It was insured that the coenzyme was not a limiting factor in the incubation mixtures by experiments in which the coenzyme level was increased with no increase in the amount of product formed.

Kinetics of the 11 β reductase system:

A. 11 β reductase activity of P₃:

1. Effect of enzyme concentration on reaction velocity.

Fig. 1 shows that the reaction velocity is proportional to enzyme concentration \underline{e} over the range $\underline{e} = 0$ to $\underline{e} = 1.0$ where $\underline{e} = 1.0$ is the arbitrary value of \underline{e} assigned to that of the standard reaction mixture.

2. Effect of time on the yield of product.

The conversion of 11-dehydrocorticosterone to corticosterone is proportional to time for at least 2-1/2 hrs as shown in Fig. II. Thus the yield of product after the arbitrary incubation period of 2 hours, used in standard conditions, serves as a basis for expressing reaction velocity \underline{v} .

TABLE II

REDUCTION AND DEHYDROGENATION AT C-11 OF VARIOUS
CORTICOSTEROIDS BY P₃ FRACTION OF MOUSE LIVER

<u>11β Reductase Activity with NADPH₂ and Fumarate</u>		
<u>Substrate</u>	<u>Product</u>	<u>Yield, μg</u>
11-dehydrocorticosterone	corticosterone	71
20 α -dihydro-11-dehydro- corticosterone	20 α -dihydro- corticosterone	30
cortisone	cortisol	49

<u>11β-HYDROXY DEHYDROGENASE ACTIVITY WITH NADP</u>		
<u>Substrate</u>	<u>Product</u>	<u>Yield, μg</u>
Corticosterone	11-dehydro- corticosterone	347
Cortisol	Cortisone	339

Conditions: 2 ml enzyme preparation + 500 μ g steroid as indicated in 0.2 ml ethanol + 65 mg niacinamide + 2 mg NADP or 2 mg NADPH₂ + 15 mg sodium fumarate, as indicated, in 6 ml Krebs-Ringer phosphate buffer, pH 7.4; incubated under N₂ at 38°C for 2 hours. Neutral chloroform extracts of incubation mixture were chromatographed on paper and zones eluted and measured spectrophotometrically.

3. Effect of substrate concentration on reaction velocity.

With the high coenzyme concentration of the standard reaction mixture constant and substrate concentration \underline{s} variable over the range $\underline{s} = 14 \times 10^{-5}$ M to $\underline{s} = 140 \times 10^{-5}$ M, the effect of \underline{s} on reaction velocity \underline{v} is shown by the Dixon-Lineweaver-Burk plot (40) in Fig. III that gives $V_{\max} = 1.6 \times 10^{-4}$ $\mu\text{moles/ml/min}$ and $K_m = 1.8 \times 10^{-5}$.

B. 11 β -hydroxy-dehydrogenase activity of P₃

1. Effect of enzyme concentration of reaction velocity.

Fig. IV shows that reaction velocity for the dehydrogenation is proportional to enzyme concentration \underline{e} over the range $\underline{e} = 0$ to $\underline{e} = 1.0$. where $\underline{e} = 1.0$ is the arbitrary value of \underline{e} assigned to that of the standard reaction mixtures.

2. Effect of time on the yield of product.

The conversion of corticosterone to 11-dehydrocorticosterone is proportional to time for at least 2-1/2 hours as shown in Fig. V. Thus the yield of product after the arbitrary incubation period of 2 hours used in standard conditions serves as a basis for expressing reaction velocity \underline{v} .

3. Effect of substrate concentration on reaction velocity.

With an excess of coenzyme, the reaction mixture constant and substrate concentration \underline{s} variable over the range $\underline{s} = 1.4 \times 10^{-4} \text{M}$ to $\underline{s} = 3.6 \times 10^{-4} \text{M}$ the effect of \underline{s} on reaction velocity \underline{v} is shown by the Dixon-Lineweaver-Burk plot in Fig. VI which gives $V_{\text{max}} = 2.7 \times 10^{-3} \text{ } \mu\text{moles/ml/min}$ and $K_m = 1.7 \times 10^{-4}$.

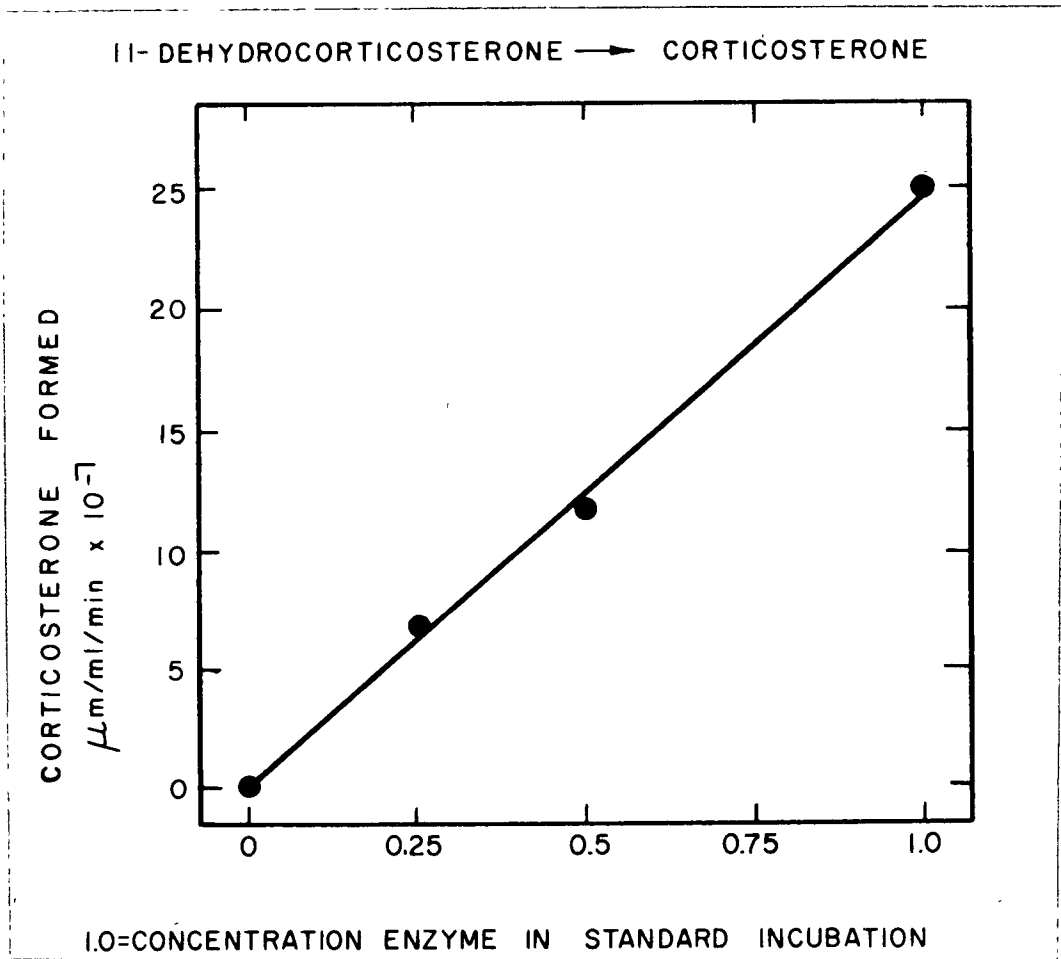


FIGURE I

Effect of enzyme concentration on reaction velocity.

Standard conditions (see text) over a range of $\underline{e} = 0$ to $\underline{e} = 1.0$ where $\underline{e} = 1.0$ is the arbitrary value of \underline{e} assigned to that of the standard reaction mixture.

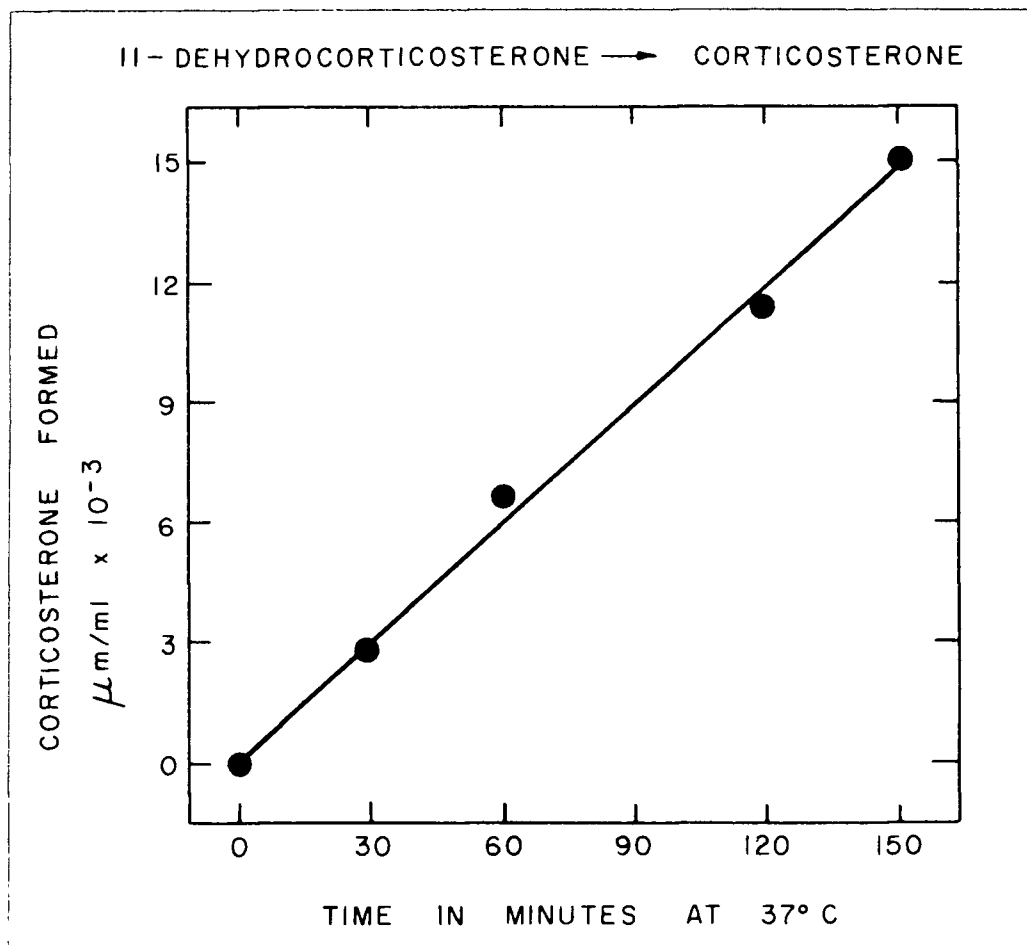


FIGURE II

Effect of time on the yield of corticosterone.

The enzyme was incubated under standard conditions (see text) over periods up to 2-1/2 hours.

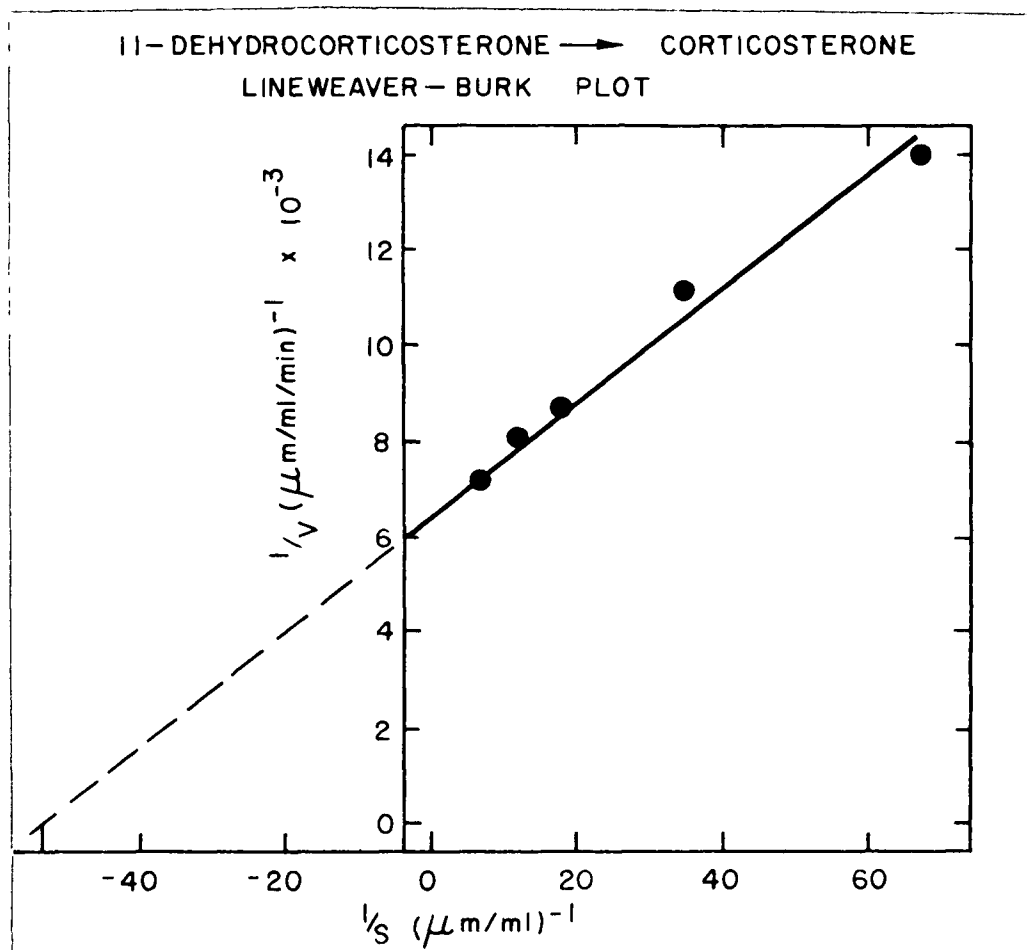


FIGURE III

Effect of substrate concentration on reaction velocity.

The enzyme was incubated under standard conditions (see text) over a substrate concentration range of $\underline{s} = 14 \times 10^{-5} \text{M}$ to $\underline{s} = 140 \times 10^{-5} \text{M}$.

$$V_{\text{max}} = 1.6 \times 10^{-4} \text{ } \mu\text{moles/ml/min.}$$
$$K_{\text{m}} = 1.8 \times 10^{-5}$$

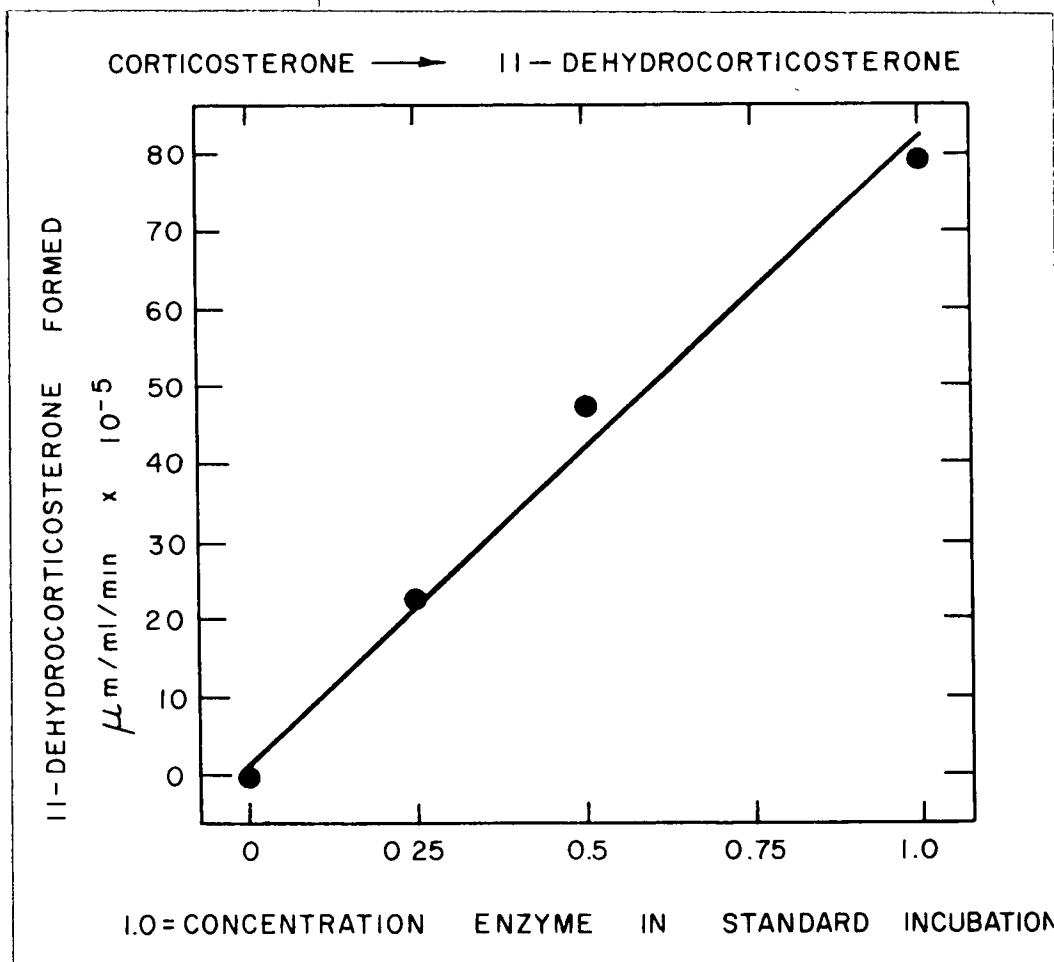


FIGURE IV

Effect of enzyme concentration on reaction velocity.

The enzyme was incubated under standard conditions (see text) over a range of $\underline{e} = 0$ to $\underline{e} = 1.0$ where $\underline{e} = 1.0$ is the arbitrary value of \underline{e} assigned to that of the standard reaction mixture.

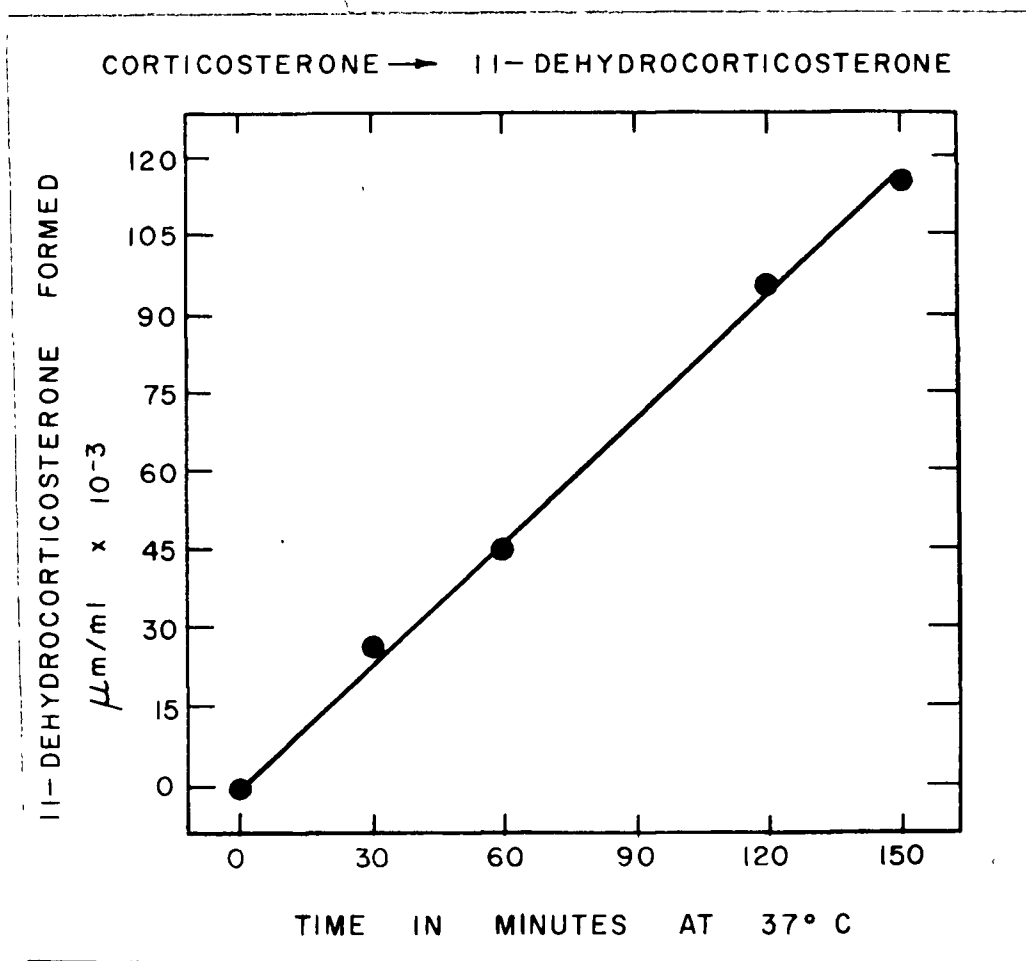


FIGURE V

Effect of time on the yield of 11-dehydrocorticosterone

The enzyme was incubated under standard conditions (see text) over periods up to 2-1/2 hours.

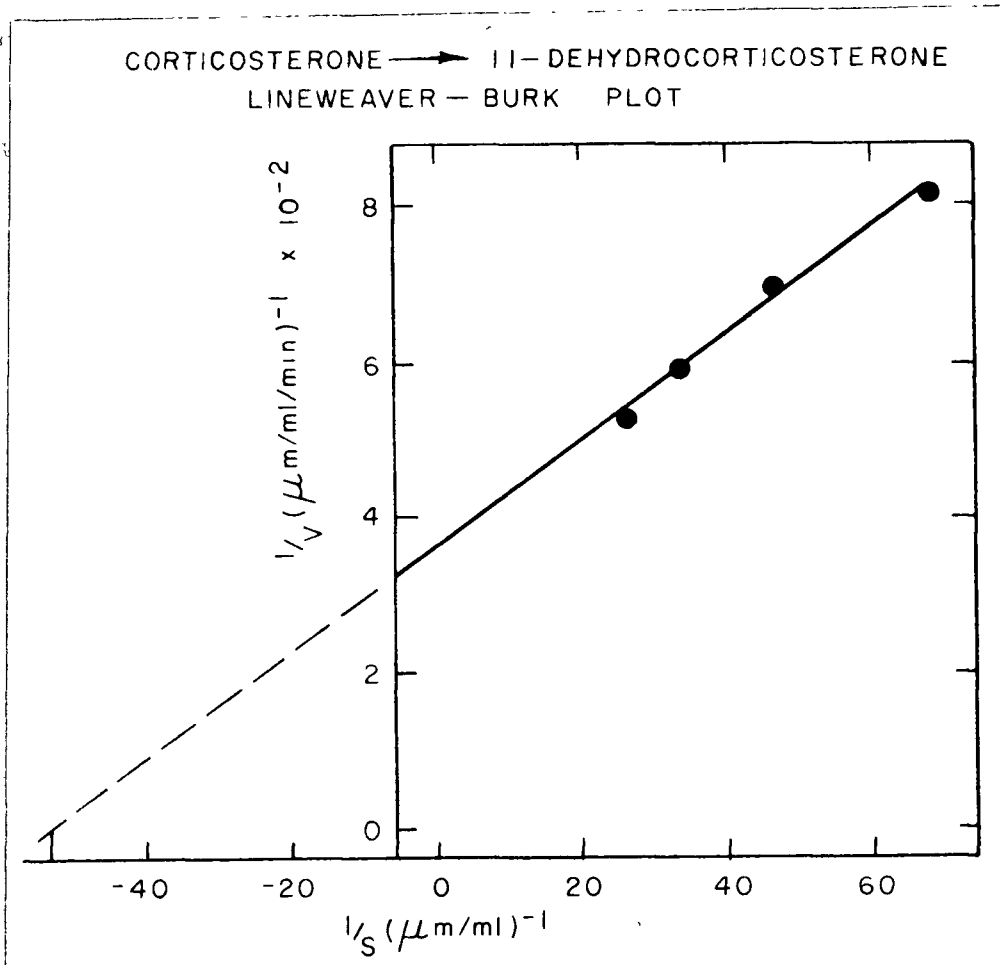


FIGURE VI

Effect of substrate concentration on reaction velocity

The enzyme was incubated under standard conditions (see text) over a substrate concentration range of $\underline{s} = 1.4 \times 10^{-4} \text{M}$ to $\underline{s} = 3.6 \times 10^{-4} \text{M}$

$$V_{\text{max}} = 2.7 \times 10^{-3} \text{ } \mu\text{moles/ml/min.}$$

$$K_m = 1.7 \times 10^{-4}$$

D I S C U S S I O N

It was observed by Southcott et al (1) that infused 11-dehydrocorticosterone disappeared rapidly from the blood of mice and a related appearance of corticosterone occurred which reached maximum values at 30 minutes post infusion. No 11-dehydrocorticosterone could be detected in blood taken later than 5 minutes after its infusion and at no time could this compound be found in the liver. The decay curve for corticosterone in liver was essentially the same as found previously following administration of corticosterone. It should be noted that no trace of 20 α dihydro-11-dehydrocorticosterone was found in either blood or liver at any of the time intervals studied. So, although the 11-keto compound is attacked by a crude C-20 reductase preparation from mouse liver at a much greater rate than the 11 β hydroxy compound, corticosterone, in vitro, it is apparent that in vivo reduction occurs very rapidly at the C-11 keto position and prior to reduction of the C-20 keto group. (26).

Reversible interconversions of hydroxy and keto steroids have been demonstrated in other laboratories. In all cases they are catalyzed by a class of widely distributed, pyridine nucleotide-requiring hydroxy-steroid dehydrogenases which are specific with respect to the position and steric configuration of the group undergoing reaction. (23,24,27,17).

The P₃ liver fraction used in these experiments was the precipitate after centrifugation at 100,000 G for one hour of the supernatant from a 6,000 G centrifugation for 20 minutes. This has been called the microsomal fraction (32) and this is the fraction of mouse liver responsible for interconversion of 11-hydroxy and 11-keto functions of corticosteroids.

Isolation of this activity in a microsomal fraction is in agreement with the results of other workers. (23,24,32). Hurlock and Talalay report the localization in mammalian and rat liver microsomes of an 11 β hydroxysteroid dehydrogenase catalyzing the reversible interconversion of cortisol and cortisone. Mahesh and Ulrich studied the metabolism of cortisol at C-11 by rat kidney and here again the microsomal fraction contained a large part of the activity. A corticosteroid 11 β -hydroxy-dehydrogenase was isolated from the microsomal fraction of human liver and placenta by Meigs and Engel.

The mouse liver 11 β reductase shows a greater coenzyme specificity than these similar microsomal enzymes isolated from other organs and species. In this case NADP and NADPH₂ activity was shown to be 8 times that of NAD and NADH₂. For the human enzyme NAD or NADP functioned approximately equally. (32). The kidney enzyme could also function with either cofactor. (24).

As shown in Table 11, although the enzyme is able to interconvert cortisone and cortisol as well as corticosterone and 11-dehydrocorticosterone, the equilibrium lies far on the side of the 11 oxidized compound. Thus this may be considered a reversible reaction with the direction of the reaction dependent on the concentration of reduced or oxidized NADP. However, the complete absence from liver of 11-dehydrocorticosterone or its 20 α dihydro derivative is consistent with the concept that liver, because of its relatively high concentration of reduced NADP (40,41,42) yields reduced metabolites as primary products of reversible coenzyme dependent dehydrogenase reactions. In extrahepatic tissues (21) where the coenzyme is predominantly in the oxidized form oxidation at the C-11 hydroxyl of corticosterone and cortisol has been demonstrated to occur very rapidly. This would explain the high ratio of 11 keto to 11 β hydroxy metabolites found in all tissues other than liver.

The enzyme performing this reaction has been named as a dehydrogenase rather than a reductase because of the reaction equilibrium which is in the direction of dehydrogenation in all tissues. Liver is the only tissue with the required coenzyme predominantly in reduced form.

The ability of this mouse liver enzyme preparation to reduce a variety of substrates suggests a lack of substrate specificity similar to the microsomal enzyme systems of rat kidney and liver, and of human liver and

placenta. It remains to be established whether single or multiple 11-dehydrogenase systems are involved.

The more specific dependence of the mouse enzyme on NADP is different from the similar enzyme in other tissues and species where NAD and NADP are almost equally effective. However, comparison of other properties of this 11 β reductase system shows them to be similar to the others mentioned: all show some activity with NAD, act with more than one substrate, are found in microsomal preparations and are reversible.

This series of kinetic data presented above is the first attempt at kinetic characterization of a corticosteroid 11 β hydroxy-dehydrogenase. The results show that in the presence of excess coenzyme equilibrium is in favour of the dehydrogenation reaction. The enzymatic nature of the reaction in both the reducing and oxidizing reactions was established. Requirement for a specific coenzyme could be demonstrated.

The reaction velocity is proportional to enzyme concentration and linear with time over the 2-1/2 hour period studied. Substrate concentration curves were also determined enabling calculation of K_m and V_{max} for reduction of the C-11 ketone and oxidation of the C-11 β hydroxyl function.

It is generally accepted that the 11 β hydroxyl is the biologically active hormone (12) so interconversion

of the 11 β hydroxyl and 11 ketone is of the utmost importance. Interconversion occurs in kidney (29), muscle (21), placenta (32), fibroblasts (30), and lymphocytes (43). In all the latter instances conditions favour dehydrogenation and only in liver in vivo does reduction appear favoured.

Efficient reduction by the liver serves to maintain steroid in the reduced condition. Other liver enzymes producing the biologically inactive products- ring A reductases and side chain reductases- are more active against 11 keto forms of the corticosteroids than the 11 β hydroxyl. (26). Therefore, because 11 β reduction seems to occur first in liver, preceding other alterations, a physiological role is suggested. This can be interpreted as a tendency to economize on the destruction of steroids by offsetting inactivation by the other liver enzymes and also the dehydrogenation which occurs in extrahepatic tissues.

S U M M A R Y

The in vivo observation that the first step in metabolism by the mouse of intravenously injected 11-dehydrocorticosterone is reduction at the C-11 position to the 11 β hydroxyl function, lead to the present studies.

This reductase activity was localized in the microsomal fraction of liver which contains an enzyme system catalyzing the interconversion of the 11 keto and 11 β hydroxyl functions of corticosterone, 11-dehydrocorticosterone, 20 α dihydro 11-dehydrocorticosterone, cortisone and cortisol.

A relative specificity for NADP and NADPH₂ as cofactors in dehydrogenation and reduction respectively can be demonstrated. Each catalyzed 8 times the activity observed with NAD or NADH₂.

The direction of the reaction is apparently controlled by the reduced or oxidized state of the coenzyme.

Certain kinetic properties of the reaction have been investigated. The reaction velocity is proportional to enzyme concentration and linear with time over the 2-1/2 hour period studied. For the reduction reaction with 11-dehydrocorticosterone as substrate $K_m = 1.8 \times 10^{-5}$ and $V_{max} = 1.6 \times 10^{-4}$ μ moles/ml/min. In the dehydrogenation reaction with corticosterone as substrate $K_m = 1.7 \times 10^{-4}$ and $V_{max} = 2.7 \times 10^{-3}$ μ moles/ml/min.

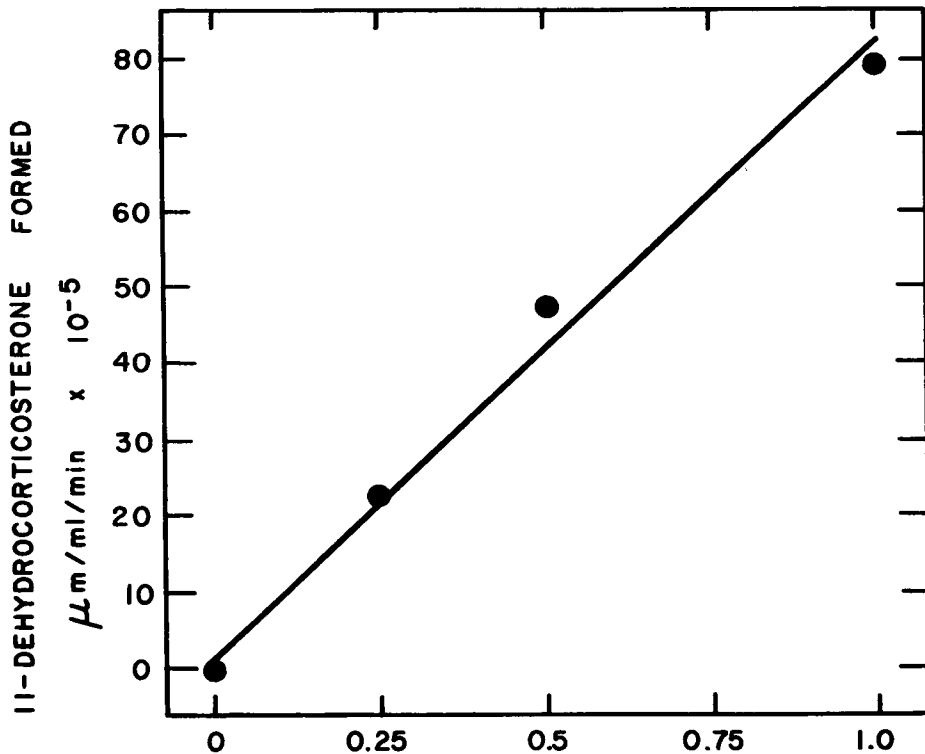
B I B L I O G R A P H Y

1. Darrach, M., In "Mechanisms of Hypersensitivity", Little Brown & Co., Boston, 613, (1959).
2. Caspi, E., Levy, H. and Hechter, O., Arch. Biochem. and Biophys., 45, 169 (1953).
3. Miller, L. and Axelrod, L., Metabolism, 3, 438 (1954).
4. Hechter, O., Solomon, M., Marchi, J., Caspi, E. and Feinstein, M., J. Clin. Endocrinol. and Metabolism, 12, 935 (1952).
5. Ingle, D., Adrenal Cortex III, Josiah Macy Jr., Found. Conf., 107 (1951).
6. Bush, L., and Mahesh, V., Biochem. J., 71, 718 (1959).
7. Wilson, H., Fairbanks, R., Scialahba, D., J. Clin. Endocrinol. and Metabolism, 14, 814 (1954).
8. Hechter, O., Frank, E., Caspi, E. and Frank, H., Endocrinology, 60, 105 (1957).
9. Schneider, J., and Horstman, P., J. Biol. Chem., 196, 629 (1952).
10. Doughaday, W., Physiol. Reviews, 39, 885 (1959).
11. Doughaday, W., J. Clin. Invest., 37, 519 (1958).
12. Sarret, L., Annals of the N. Y. Academy of Science, 82, 802 (1959).
13. Forchelli, I., and Dorfman, R., J. Biol. Chem., 223, 443, (1956).
14. Ulrich, F., Biochem. J., 61, 361 (1958).
15. Hubener, H. J., Fukushima, D. K., and Gallagher, F., J. Biol. Chem., 220, 449 (1956).
16. Bush, I. and Mahesh, V., Biochem. J., 71, 705 (1959).

17. Axelrod, L., and Miller, L., Arch. Biochem. and Biophys., 60, 373 (1956).
18. Talalay, P., and Williams-Ashman, H., Proc. Natl. Acad. Sci. (Wash.), 44, 15 (1958).
19. Villee, C., J. Biol. Chem., 215, 171 (1955).
20. Glenn, E., Stafford, R., Lister, S., and Bowman, B., Endocrinology, 61, 128 (1957).
21. Sweat, M., and Bryson, M., Biochim. et Biophys. Acta., 44, 217 (1960).
22. Dougherty, L., Adrenal Cortex II, Josiah Macy Jr., Found. Conf., 88 (1950).
23. Hurloch, B. and Talalay, P., Arch. Biochem. and Biophys., 80, 468 (1959).
24. Mahesh, V., and Ulrich, F., J. Biol. Chem., 235, 356 (1960).
25. Palade, G., and Siekewitz, P., J. Biophysical and Biochemical Cytology, 2, 671 (1956).
26. Darrach, M., Krehbiel, R. and Burton, A. F., unpublished work.
27. Roberts, S., and Szego, E., Ann. Rev. Biochem, 24, 543 (1959).
28. Fish, A., Hayano, N., and Pincus, G., Arch. Biochem. and Biophys., 42, 480 (1953).
29. Ganis, F., Axelrod, R., Miller, L., J. Biol. Chem., 218, 841 (1956).
30. Dougherty, T., "The Leukemias", Acad. Press., N.Y., (1957).
31. Engel, L., and Langer, L., Ann. Rev. Biochem., 30, 449 (1961).
32. Meigs, R., and Engel, L., Endocrinology, 69, 152 (1961).

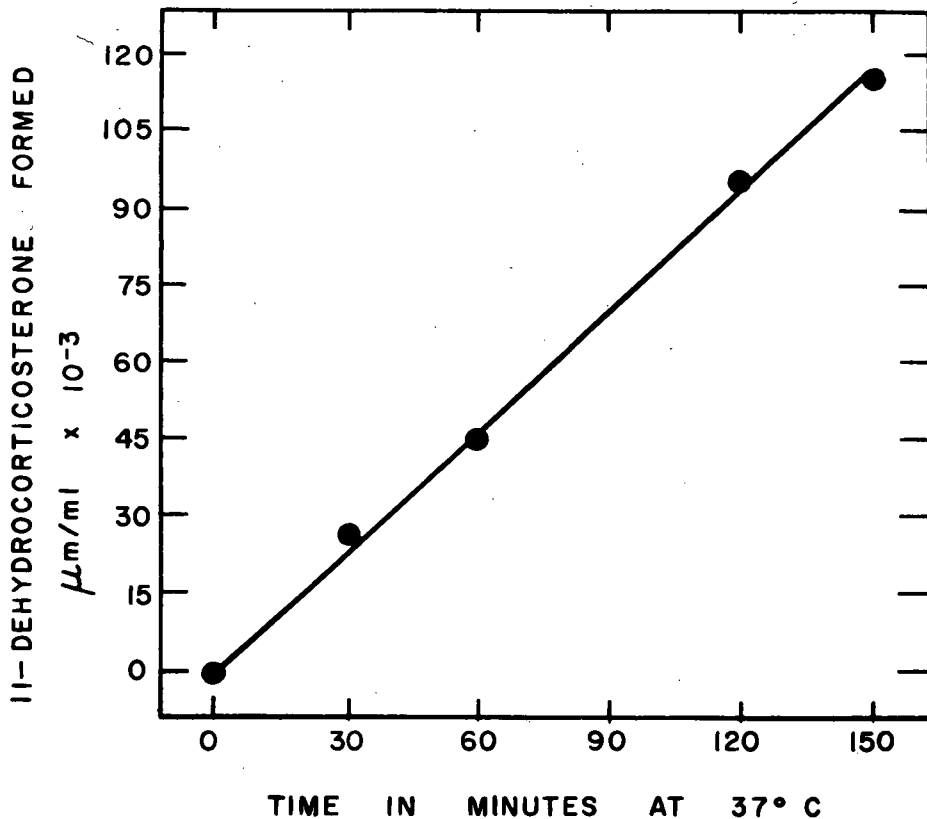
33. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, 34, 442 (1940).
34. Hogeboom, G., and Schneider, W. C., In "The Nucleic Acids", Vol. II, Chapt. 21, Ed. Chargaff, E., and Davidson, J. N., N. Y., Acad. Press (1955).
35. De Courcy, C., and Schneider, J. J., *J. Biol. Chem.*, 223, 885 (1956).
36. Zaffaroni, A., and Burton, R. B., *J. Biol. Chem.*, 193, 749 (1951).
37. Mader, W. J., and Buck, R. R., *Analytical Chemistry*, 24, 666 (1952).
38. Zaffaroni, A., *J. Am. Chem. Soc.*, 72, 3828 (1950).
39. Romanoff, L. P., Marris, C. W., Welch, P., Rodriguez, R., and Pincus, G., *J. Clin. Endocrinol. and Metabolism*, 21, 1413 (1961).
40. Dixon, M., and Webb, E. C., "Enzymes," Longmans, London (1960).
41. Bassham, J. A., Birt, L. M., Hems, R., and Loening, V. E., *Biochem. J.*, 73, 491 (1959).
42. Silber, R., Gabrio, W. G., and Huennekens, F. M., *J. Clin. Invest.*, 41, 230 (1962).
43. Dougherty, J. F., Berliner, M. L., and Berliner, D. L., *Endocrinology*, 66, 550 (1960).
44. Mahesh, V., and Ulrich, E., *Nature*, 184, 1147 (1959).

CORTICOSTERONE → 11-DEHYDROCORTICOSTERONE

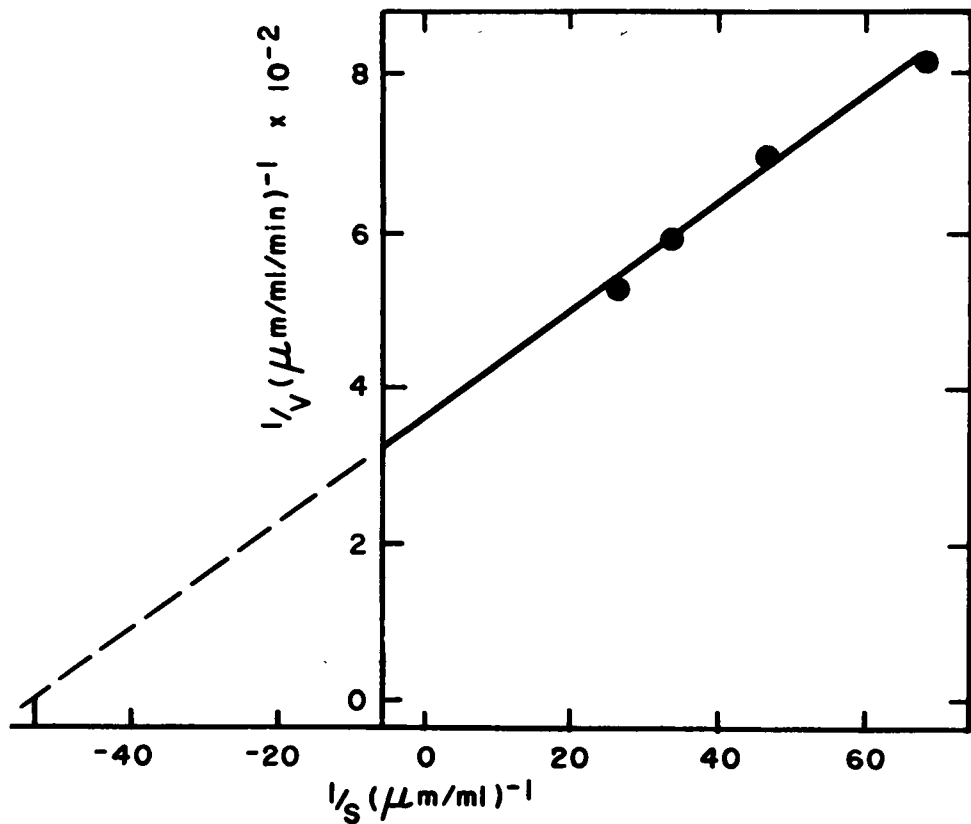


I.O = CONCENTRATION ENZYME IN STANDARD INCUBATION

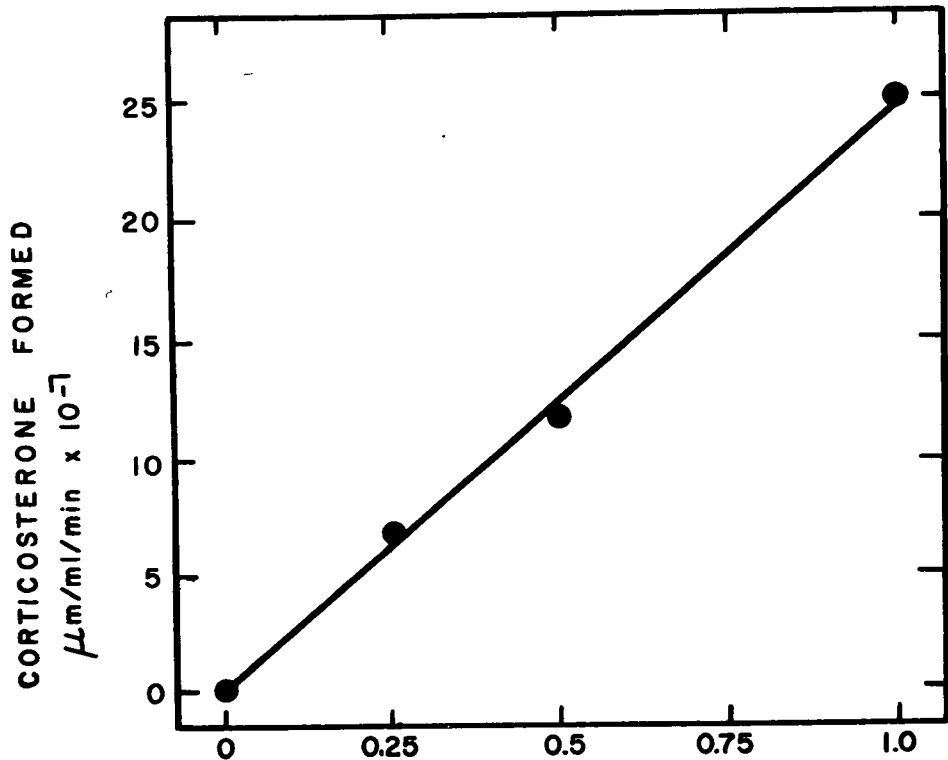
CORTICOSTERONE \rightarrow 11-DEHYDROCORTICOSTERONE



CORTICOSTERONE \rightarrow 11-DEHYDROCORTICOSTERONE

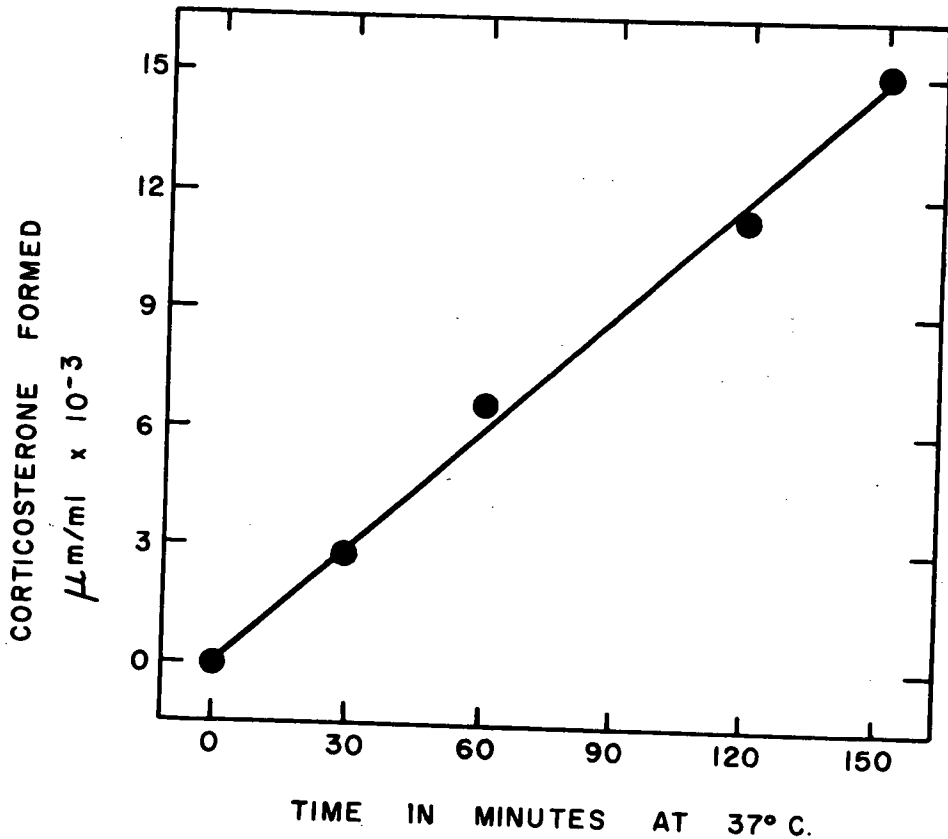


11-DEHYDROCORTICOSTERONE → CORTICOSTERONE



1.0=CONCENTRATION ENZYME IN STANDARD INCUBATION

11- DEHYDROCORTICOSTERONE \rightarrow CORTICOSTERONE



11-DEHYDROCORTICOSTERONE \rightarrow CORTICOSTERONE

