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Enzymatic Degradation of Side-Chain of Adrenocortical Steroids: Conversion of Hydrocortisone to Reichstein's E.* (22254)

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(Introduced by George Sayers.)

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In recent publications from this laboratory (1,2) an enzyme system found in both microsome and supernatant fractions of rat liver has been described which induces an alteration in the C-20 ketone group of adrenocortical steroids. Further observations on this system have been made and in addition positive evidence has been obtained that the reaction involving the C-20 ketone of the steroid molecule is reductive in nature.

Methods. Methods used in this study were identical with those described in previous publications(1,2).

Results. The first study in this series was undertaken to determine if the system reducing ring A could be separated from the system reducing the 20-ketone. A 0.25 *M* sucrose homogenate of rat liver free of nuclei and mitochondria was centrifuged at 20,000 X gravity for one hour. The supernatant fraction was discarded and the microsome fraction was resuspended and recentrifuged twice under the same conditions. Results obtained following incubation (37°C, 1 hr) of cortisone with the twice-washed microsome fraction are indicated in Fig. 1. It may be noted that the extent of Δ^4-3-ketone reduction in the complete system was only slightly less than that of 20-ketone reduction. Subsequent studies have confirmed this observation.‡ These results are at variance with those of Tomkins and Isselbacher(3) who reported that the Δ^4-3 ketone reducing system is present only in the supernatant fraction of rat liver.

p-Chloromercuribenzoic acid (PCMBA) inhibition of the Δ^4-3 ketone reducing system and of the 20-ketone reducing system in the microsome fraction was investigated. Fig. 2

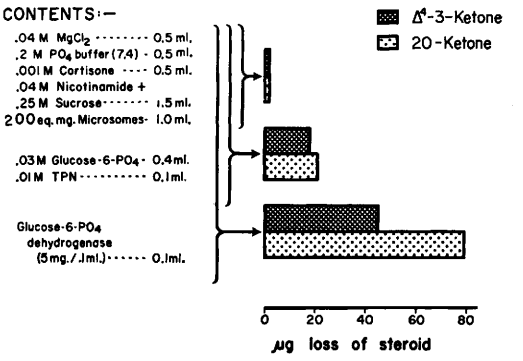

FIG. 1. Presence of both C-20 ketone and Δ^4-3-ketone reducing systems in microsome fraction of rat liver. See footnote (§) for definition of equivalent milligram (eq mg).

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† Data submitted in this report are in partial fulfillment for the Ph.D. degree. Predoctoral Fellow of the Arthritis and Rheumatism Foundation, 1955-1956. Present address: Upjohn Co., Kalamazoo, Mich.

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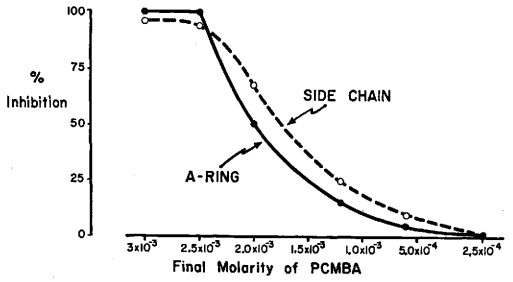

FIG. 2. Inhibition of Δ^4-3-ketone and C-20 ketone reducing systems by p-chloromercuribenzoate. Conditions as in Fig. 1.

indicates that inhibition of both systems was virtually complete at a final concentration of 3.0×10^{-3} *M* PCMBA. However, the inhibition by PCMBA appears to be due to inhibition of the TPNH generating system, since it was shown that glucose-6-phosphate dehydrogenase was strongly inhibited by PCMBA (Table I). The question of a dependence of C-20 and Δ^4-3-ketone reduction on SH-enzymes must therefore be considered as unsettled.

The enzymatic complexity of the microsome fraction with respect to reduction of steroid ketones is illustrated by the data of Fig. 3. In this study, equal quantities of cortisone, hydrocortisone, 11-desoxycortisone, tetrahydrocortisone, dehydroepiandrosterone, and androstenedione were incubated at 37°C for 2 hours using the conditions described in Fig. 1. Four-hundred equivalent milligrams§ of microsomes were used as source of enzyme. Loss of the C-20 group was followed by the Porter-Silber reaction(4). The Callow modification of the Zimmerman reaction(5) was

TABLE I. Inhibition of Glucose-6-Phosphate Dehydrogenase by p-Chloromercuribenzoate.

	E_{340}*
Complete system	.465
" " + 1×10^{-3} *M* PCMBA	.061
" " + 2 " "	.011
" " + 3 " "	.035

* Optical density reading at 340 mμ one min. after start of reaction.
Conditions: Sigma Chemical Co. Bulletin No. 201, Nov. 1951.

§ An equivalent mg of microsomes represents all of the microsome fraction obtained under prescribed centrifugation conditions from one mg wet weight of whole liver.

used to follow disappearance of the 17-ketone group. The steroids employed have a variety of biological actions. Tetrahydrocortisone and 11-desoxycortisone are biologically inactive. It is apparent that the metabolism of these steroids is not related in a simple and obvious way to their metabolic effects. The metabolism of typical 17 ketosteroids (dehydroepiandrosterone, androstenedione) in the microsome fraction adds to the complexity. The fact that tetrahydrocortisone reacts to a greater extent than the other steroids tested was confirmed by additional investigations (Fig. 4). During the first 90 minutes of in-

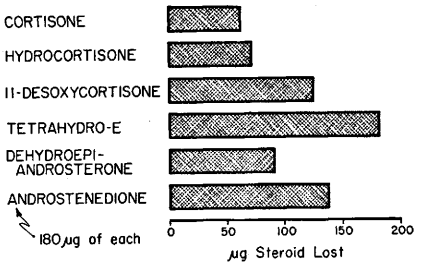

FIG. 3. Enzymatic complexity of rat liver microsomes with respect to reduction of steroid ketone groups.

cubation the loss of the 20-ketone of tetrahydrocortisone was about twice the rate observed for hydrocortisone. These *in vitro* observations reflect the *in vivo* observations of others, in which it was shown that tetrahydrocortisone disappears from the blood faster than hydrocortisone when administered to experimental subjects(6). Also, studies of Sandberg *et al.*(7) indicate that cortisone disappears at a more rapid rate from the blood than hydrocortisone, and that the steroid degradation products of hydrocortisone appear in the urine at a less rapid rate than the degradation products of cortisone. Whether the metabolic rate studies recorded in Fig. 4 represent the enzymatic basis for the *in vivo* observations cannot be stated with certainty at this time.

Isolation of Reichstein's E. Seven and two-tenths milligrams of hydrocortisone (free alcohol), dissolved in 20 ml of 0.25 *M* sucrose made to 2% with ethanol were incubated with 20 equivalent grams of rat liver microsomes (washed 2 times with 0.25 *M* sucrose). The

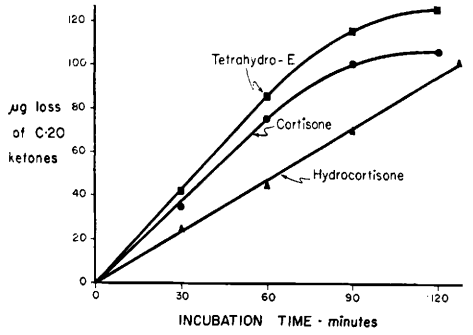

FIG. 4. Relative rates of reactions of various steroids in rat liver microsome fraction. Conditions as in Fig. 1.

following cofactors were added to the incubation medium: 14.0 ml of 0.03 *M* glucose-6-phosphate, 20.0 ml of 0.02 *M* phosphate buffer (pH 7.4), 10.0 ml of 0.04 *M* $MgCl_2$, 10.0 ml of 0.04 *M* isocitrate, 10.0 ml of 0.01 *M* TPN. The pH of the medium (7.4) was checked before incubation and 10.0 mg of glucose-6-phosphate dehydrogenase were added immediately before evacuation. The enzyme mixture was transferred to a 37°C water bath and incubated for 45 minutes. The aqueous phase was extracted 3 times with 400 ml $CHCl_3$. The $CHCl_3$ extract was dried under a stream of air and the residue redissolved in 70% ethanol and subsequently partitioned between 70% ethanol and pentane for removal of interfering substances. The pentane fraction was discarded and the 70% ethanol phase dried under vacuum. The dry residue was taken up in a small amount of chloroform and placed on a Florisil column (8). The column was eluted with 100 ml of chloroform, 100 ml of 2% methanol:chloroform, and 200 ml of 25% methanol:chloroform. The eluates were dried and the residues dissolved in methanol. One-twentieth of each eluate was applied to a paper strip and chromatographed in the benzene: 50% methanol:water system of Bush(9). Paper chromatograms were observed under ultraviolet light and subsequently sprayed with 4% ethanolic phosphomolybdic acid. Observation under the ultraviolet lamp revealed the presence in the 25% methanol-chloroform eluate of a compound more polar than hydrocortisone. The other eluates contained no detectable steroids. The phosphomolybdic acid reagent revealed (blue spot) the presence in this same eluate of a still more polar compound which was not visible under the ultraviolet lamp. In order to remove unreacted hydrocortisone the remainder of the 25% methanol-chloroform eluate was dried under vacuum and the dry residue placed on eight 1½-inch wide paper strips and chromatographed as before. The paper chromatograms were observed under the ultraviolet light. Regions which corresponded to hydrocortisone were discarded. More polar regions were eluted with methanol, dried under vacuum, redissolved, and a sample which corresponded to

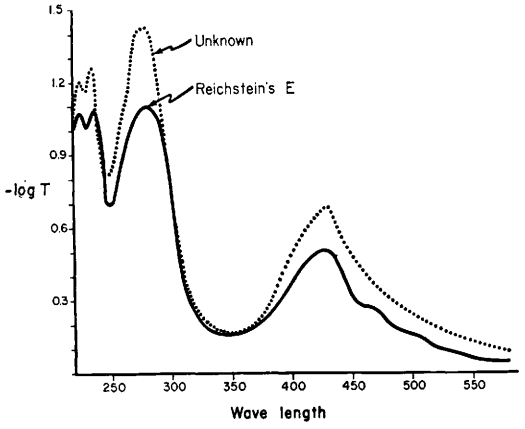

FIG. 5. Sulfuric acid induced absorption of unknown steroid compared to authentic sample of Reichstein's E.

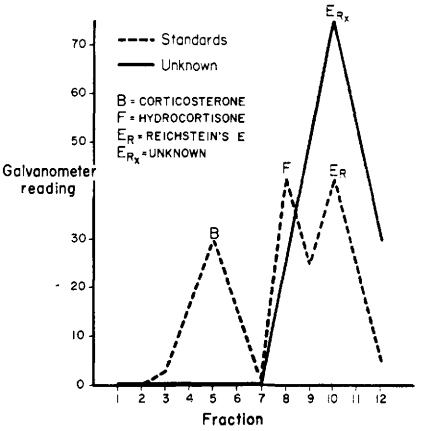

FIG. 6. Silica gel chromatography and fluorescence of unknown steroid compared to authentic sample of Reichstein's E.

one-twentieth of the original was rechromatographed in benzene:25% ethyl acetate. Ultraviolet and phosphomolybdic acid analysis revealed the presence of the 2 polar steroids as expected. In order to separate the 2 unknown steroids, the remainder of the methanol eluate was dried and rechromatographed on six 1½-inch wide paper strips in benzene:25% ethyl acetate. The unknown steroid which was not visible under the U-V lamp was not further analyzed. The areas on the 6 strips which were visible under the U-V lamp were eluted with methanol. In order to further purify the unknown steroid, the combined methanol eluates were rechromatographed on four 1½-inch wide paper strips in the benzene:25% ethyl acetate system. The ultraviolet absorbing steroid was eluted from the several strips, combined, dried under vacuum and diluted to 20.0 ml with absolute methanol.

The following tests with the recorded results indicate that the unknown compound was Δ^4-pregnene-11 β, 17 α, 20,21-tetrol-3-one (Reichstein's substance E). The stereochemical configuration of the C-20 alcohol of the experimentally derived product has not been definitively established.

Tests	Results
1. Rf values of unknown and known (free alcohols), on Bush(9) and Zaffaroni(10) systems.	1. Same Rf value for unknown and authentic sample of Reichstein's E.
2. Ultraviolet absorption spectrum at 220 to 260 mμ.	2. Absorption maximum at 240 mμ = Δ^4-3-ketone.
3. Phenylhydrazine reaction of Porter-Silber (4).	3. Unknown did not peak at 410 mμ. Not a C-20 ketone.
4. Sulfuric acid chromogen spectrum.	4. Spectrum (330 to 600 mμ). Identical with Reichstein's E. See Fig. 5.
5. Unknown chromatographed on silica gel column according to the method of Sweat(11).	5. Fluorescence and chromatographic behavior identical with Reichstein's E. See Fig. 6.
6. Unknown acetylated with pyridine-acetic anhydride 16 hr at room temp. Acetates of unknown and Reichstein's E chromatographed on Bush(9) and Zaffaroni(10) systems.	6. Rf values same for single chromatograms as well as mixed acetates.

Quantitative analysis at 240 mμ and calculation from known standards indicated that 2.1 mg of Reichstein's E were obtained from 7.2 mg of hydrocortisone. The more polar compound has not been definitely identified, but the evidence obtained suggests that it is a pentol. That the reactions in this system are probably exclusively reductive in nature was shown when 4-C^{14} hydrocortisone‖ was incubated with the preparation described, and the extracts chromatographed on the Zaffaroni system(10) for 92 hours. No C^{14} was present in the run-off from the paper strips. If oxidative reactions had occurred, 17-ketosteroids labelled with C^{14} would have been expected to appear in this fraction.

Conclusions. 1. Observations concerning the enzyme systems of the microsome fraction of rat liver which effect the C-20 ketone reduction of adrenocortical steroids have been described. 2. One product of hydrocortisone conversion in the microsome fraction of rat liver has been identified as Reichstein's E. The presence of at least one other product was noted.

We are indebted to Dr. John Schneider of the Jefferson Medical School for his generous gift of Reichstein's E, and to Dr. Constance de Courcy of the Jefferson Medical School for introducing us to the technics of the Bush system of paper chromatography.

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Steroid-Induced Adrenal-Pituitary Hypofunction II. (22255)

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It has been demonstrated that daily administration of hydrocortisone to rats lowers their tolerance to intraperitoneal injection of potassium chloride(1). Since this observation suggested adrenal-pituitary hypofunction, efforts were made to determine if these animals could be protected by treatment with various hormones. Intravenous administration of ACTH or aldosterone immediately prior to the injection of potassium chloride increased the tolerance of steroid-treated animals to KCl stress(2). It was observed that the same response was obtained if animals were given 1% NaCl to drink for 18 hours prior to injection of potassium chloride(3).

The protective action of sodium chloride suggested that the mortality of non-protected animals was not due to a hypofunctional adrenal or pituitary but rather to an electrolyte disturbance as a result of steroid treatment. The present experiments were designed (1) to measure the amount of sodium and potassium excreted in the urine during steroid administration, and (2) to determine the tolerance to potassium stress in rats depleted of sodium and potassium.

Methods. 1. Male rats, averaging 160 g body weight were placed in metabolism cages (2/cage) and fed Ingle's liquid medium carbohydrate diet *ad libitum*(4). Following a 2-week control period, these animals were injected once daily for 10 days with either 16% gelatin, 4 mg, hydrocortisone (F) in 16% gelatin, or 6 mg 11β, 17α-dihydroxy-4-pregnene-3,20-dione (21-desoxy-F) in CMC.* Urine volumes were recorded daily and urinary sodium and potassium were determined in a flame photometer. 2. Male rats were given intraperitoneal injections of 8 mg Thiomerin sodium (Wyeth), 5 mg Neohydrin (Lakeside), or 6 mg Diamox (American Cyanamid) in 0.2 cc aqueous solution. The rats were placed in metabolism cages, fasted for 18 hours and then injected with 0.16 M potassium chloride (5 cc/100 g body weight). The 18-hour urine collections were analyzed for sodium and potassium.

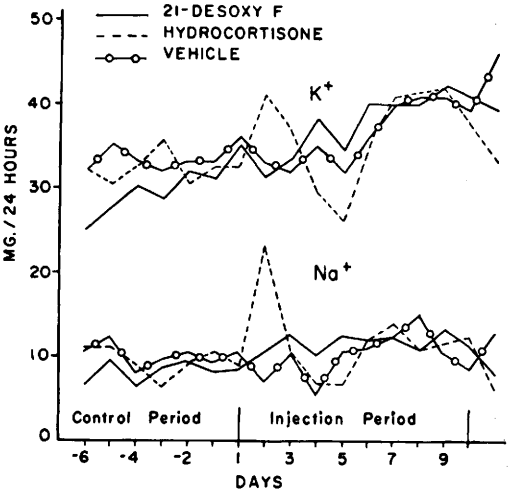

FIG. 1. Effects of hydrocortisone and 21-desoxy F on urinary sodium and potassium excretion.

* 0.5% carboxymethyl cellulose, 0.4% Tween 80, 1.5% benzyl alcohol and 0.9% sodium chloride.