Steroid Compounds Resulting from Incubation of Cortisone with Surviving Liver Slices.*† (20253)

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The major site of inactivation of steroid hormones is the liver. Catabolism of androgenic and estrogenic steroids by hepatic tissue has been demonstrated both *in vivo* and *in vitro*(1-3). The hepatic metabolism of several adrenocortical steroids has been investigated by incubating these compounds with liver slices(4). The results show that the steroids studied, including cortisone (Compound E), are catabolized by reduction of the conjugated, unsaturated bonds in ring A of the molecule and by degradation of the α-ketol side chain. These alterations in the molecular structure of cortisone result in loss of biologic activity of this hormone. Hechter (5) perfused the isolated rat liver with blood containing cortisone and observed rapid disappearance of this steroid. This observation, coupled with the clinical finding that Compound E administered orally is metabolically active, led this investigator to suggest that certain metabolites of cortisone, formed by the liver, must possess physiologic activity.

The purposes of the present investigation were to study the metabolism of cortisone by rat liver slices and to attempt identification of the compounds derived under the conditions of these experiments. The procedure followed included incubating rat liver slices in a physiologic medium containing cortisone and analyzing the resulting steroid mixture by paper chromatography. The results show that under the conditions of this experiment, two derivatives of cortisone are formed. One has been demonstrated to be Compound F and the other, which has been only partially identified, is thought to be Reichstein's Substance E.

Procedure. Rat liver slices weighing approximately 300 mg were placed in a flask containing 4.6 ml of Kreb's solution, buffered with phosphate to pH 7.4. Prior to incubation, 1.0 mg of cortisone (free alcohol) in 0.2 ml of propylene glycol was added. The flask was flooded with oxygen, stoppered, and incubated at 37° for 3 hours with occasional shaking. Control studies were done in which liver slices were incubated in the same medium but without the addition of cortisone. In addition, liver slices, which had previously been boiled in isotonic saline, were incubated in a medium containing cortisone. On completion of incubation, the steroid fraction was recovered by the following procedure. Absolute ethyl alcohol was added to the incubation mixture in amount sufficient to bring the final alcohol concentration to 70%. The mixture was allowed to stand overnight at room temperature and was filtered. The alcoholic solution was extracted 3 times with equal volumes of petroleum ether and the petroleum ether fraction discarded. The alcohol was removed by distillation *in vacuo* at a temperature of 45°C. The aqueous solution was extracted 3 times with equal volumes of ethylene dichloride, the latter fraction dried over sodium sulfate, and the ethylene dichloride removed under vacuum. The residue containing the steroids was dissolved in 1.0 ml of an equal mixture of chloroform and methyl alcohol and applied to the paper strip. Chromatography was done by the method of Zaffaroni(6) using propylene glycol and toluene as solvents. The steroids present on the chromatogram were detected by use of the alkaline silver nitrate reagent and by exposing the paper to ultraviolet light (2537A). Steroid compounds which reduce alkaline silver nitrate have an alpha-ketol side chain(6). Those steroids with conjugated, unsaturated bonds in ring A absorb ultraviolet light(7).

Identification of steroid compounds isolated. A variety of physical and chemical procedures

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were used in an attempt to determine the molecular configuration of the steroids isolated in this experiment. The following procedures were adapted for use in efforts to identify the 3 substances obtained. 1) *Ultraviolet absorption spectra.* A sample of each compound was dissolved in methyl alcohol and the absorption spectrum was determined from 226 to 250 mμ. Peak absorption at 240 mμ was considered evidence of an alpha-beta unsaturated ketone group(8). 2) *Absorption spectra of the sulfuric acid derivatives.* The steroids isolated in this experiment were treated with concentrated sulfuric acid and the spectra were compared with those obtained from the addition of sulfuric acid to crystalline Compound E and Compound F. Comparison was also made with spectral analyses of related steroids recorded in the literature(9). 3) *Comparison of chromatographic mobility of unknown steroids with that of crystalline compound E and compound F.* Aliquots of the unknown steroids were chromatographed with crystalline samples of cortisone or Compound F. 4) *Color reactions.* The unknown substances present on the chromatograms were tested with certain color reagents as follows: a) Alkaline silver nitrate (6), b) Iodine reagent(8), c) Concentrated sulfuric acid(8).

Results. Control observations demonstrate that no steroid compound was present on the chromatogram when the incubation medium did not contain cortisone (Fig. 1, A). In addition, a single band identified as cortisone was obtained on chromatograms when liver slices had been previously inactivated by boiling and were incubated in a medium containing cortisone (Fig. I, B).

In all other experiments, when liver slices were incubated in a medium containing cortisone, 3 distinct fractions were detected (Fig. 1, C). For purposes of discussion, these are designated as incubation products I, II, and III in descending order. Areas of the paper chromatogram containing these products were separately eluted and efforts to identify each substance were made by the procedures described above.

In all experiments, incubation product III was obtained in greatest amount as judged by

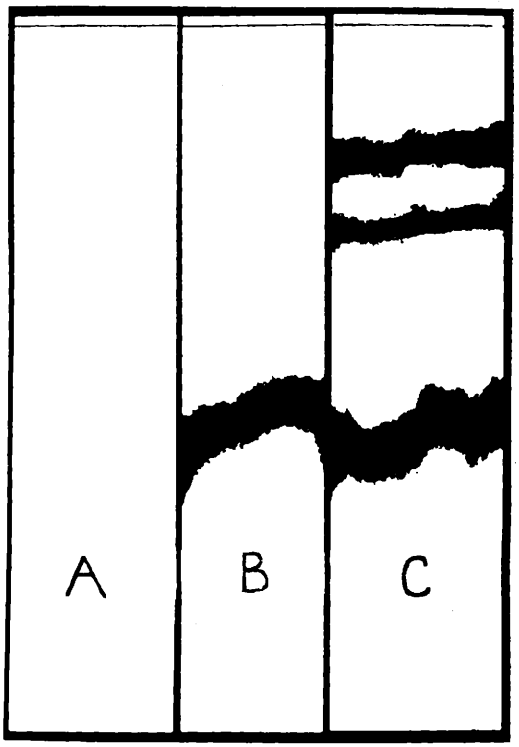

FIG. 1. Steroid compounds obtained on paper chromatograms. Dark bands represent steroids which absorb ultraviolet light. A. Chromatogram of surviving liver slices incubated in a medium without cortisone. B. Chromatogram of inactivated liver slices incubated in a medium containing cortisone. C. Chromatogram of surviving liver slices incubated in a medium containing cortisone.

size and intensity of the band when the paper chromatogram was visualized under ultraviolet light. The following results establish the identity of this compound as cortisone: 1) a positive reaction for an alpha-ketol group when tested with alkaline silver nitrate, 2) an intense blue color with the iodine reagent, 3) maximum ultraviolet absorption at 240 mμ, 4) chromatographic mobility identical with that of crystalline cortisone, and 5) a sulfuric acid derivative with absorption maxima at 280, 345, and 415 mμ. These absorption peaks coincide with those of the sulfuric acid derivative of crystalline cortisone.

Incubation product II was proven to be Compound F. Results of procedures designating the molecular structure of this steroid included: 1) a positive reaction for an alpha-ketol side chain with alkaline silver nitrate, 2) production of a compound with yellow-

green fluorescence when treated with concentrated sulfuric acid, 3) maximum absorption at 240 mμ in the ultraviolet, 4) chromatographic mobility identical with that of compound F in mixed chromatograms, and 5) addition of sulfuric acid produced a colored product with absorption maxima at 280, 395, and 475 mμ. This is identical with the spectrum derived from crystalline Compound F.

Total identification of the molecular configuration of incubation product I was not achieved largely because the quantity of this material obtained was limited. The data available showed that 1) this compound did not reduce alkaline silver nitrate and, thus, does not possess an alpha-ketol side chain; 2) a maximum absorption at 240 mμ indicated that the alpha-beta unsaturated ketone was present; 3) the chromatographic mobility was less than that of cortisone or Compound F. The decreased mobility of this product as compared to Compound F could be accounted for either by reduction of the ketone group on the carbon atom at position 20 to an alcohol or by the addition of another atom of oxygen to the molecule. It is suggested but not established that the substance is Reichstein's Substance E (Δ4-pregnene-11 β, 17 α, 20, 21 tetrol-3-one) and is formed from cortisone by reduction of the ketone groups at C-11 and C-20.

Discussion. The data presented by Schneider and Horstman(4) show that liver slices alter the molecular structure of cortisone by reduction of the conjugated, unsaturated bonds in ring A and by degradation of the side chain at C-17. The studies reported here did not concern these changes but demonstrate that rat liver slices may also metabolize cortisone by reducing the ketone groups at C-11 and probably at C-20. Conversion of the ketone at position 11 to an hydroxyl group results in the formation of Compound F. If both the carbonyl groups at C-11 and C-20 are reduced the steroid formed is Reichstein's Substance E. This compound has been isolated from the adrenal cortex(10).

It has been demonstrated that conversion of Compound E to Compound F occurs *in vivo* and it has been suggested that this may account for some of the metabolic activity previously attributed to cortisone. Burton, Keutmann, and Waterhouse(11) administered cortisone acetate to normal humans and patients with Addison's disease and recovered increased amounts of cortisone, Compound F, tetrahydrocortisone, and a fourth steroid from the urine. Hechter(5) has pointed out that although cortisone is rapidly destroyed by the liver, the metabolic activity following oral administration of this steroid persists for some time. This investigator has suggested that certain metabolites of cortisone formed in the liver are physiologically active. Ingle(12) has observed that cortisone does not exert its usual effect on carbohydrate tolerance in the hepatectomized rat, whereas the administration of aqueous adrenal cortex extract results in a decrease in glucose tolerance. Since adrenal cortex extract contains a variety of steroid hormones, including Compound F, this observation seems to indicate that cortisone must be altered by passage through the liver before it can affect carbohydrate metabolism.

These observations taken together with the experimental data reported here which demonstrate that cortisone is partially converted to Compound F by liver slices suggest that some of the metabolic activity which in the past has been attributed to Compound E may actually be dependent on its conversion to Compound F.

Summary. 1. Liver slices were incubated in a physiologic, buffered medium containing cortisone. Following incubation, the steroid fraction was recovered and the individual steroids isolated by paper chromatography. 2. Under the conditions of this experiment, 3 steroids were isolated on the paper chromatogram. The compound of greatest mobility and obtained in largest amount was cortisone, unaltered by incubation. The product of intermediate mobility was identified as Compound F. The least mobile compound was not identified. 3. On the basis of observations in the literature and the experimental data reported here, it is suggested that the metabolic activity of cortisone may actually be dependent on its conversion to Compound F.

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Some Effects of Desoxycorticosterone Glycoside on Chick Fibroblasts *in vitro.** (20254)

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Although desoxycorticosterone is known to elicit a definite response from connective tissues when injected into the intact body, the effect of this substance upon connective tissues *in vitro* is comparatively little known. Adrenal cortical extracts have been reported by von Haam and Cappel(1), to prolong the survival of fibroblasts grown in Carrel flasks. Using desoxycorticosterone at concentrations of 0.03 mg/ml, Cornman(2) showed that there was a reversible inhibition of the beat of heart fragments taken from chick embryos; he also showed that fibroblasts are selectively damaged in mixed cultures of fibroblasts and endothelium grown in roller tubes. The investigation reported here was undertaken to study the effects of low concentrations of desoxycorticosterone on the rate of growth and the morphology of cells in tissue culture *in vitro.*

Materials and procedures. Stock cultures of connective tissue cells in a medium of chicken plasma and embryo extract were derived from explanted fragments of 7-day-old chick femurs. The cultures were cut into fragments as nearly equal in size as possible. One fragment was explanted into a medium consisting of equal parts of heparinized chicken plasma and chick embryo extract, the latter containing varying amounts of desoxycorticosterone glycoside.† The control fragment was explanted into the same medium without the desoxycorticosterone. The final concentrations of the desoxycorticosterone glycoside in the medium were: 30, 15, 7.5, 3.5, and 2 μg/ml. For each concentration about 20 pairs of cultures were made. Some other cultures were studied with this steroid at concentrations up to 240 μg/ml. The areas of outgrowth of the cultures were measured after 48 hours and the relative increases treated statistically(4). At the same time, photomicrographs were taken of cells in selected pairs of cultures to record morphological changes.

Results. Concentrations of desoxycorticosterone glycoside above 30 μg/ml were found to decrease the growth rate of tissue cultures of chick embryo fibroblasts. Below this concentration the steroid increases the rate of growth of fibroblasts. The stimulation of the growth rate of fibroblasts increases with decreasing concentration until a maximum stimulation is reached at 7.5 μg/ml. Below this concentration, the stimulation decreases until, at 2 μg/ml it is no longer measurable by these methods. The average differences in the relative growth of experimental and con-

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