ISOLATION OF 17-HYDROXYCORTICOSTERONE FROM THE URINE IN A CASE OF CUSHING'S SYNDROME ASSOCIATED WITH SEVERE DIABETES MELLITUS

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Many of the features of Cushing's syndrome are those that would be expected to result from an oversupply of adrenal cortical hormones. Indeed, Albright (1) has attributed the development of this syndrome to overproduction by the adrenal cortex of the carbohydrate-active hormones (S hormone), while Kenyon (2) and Kepler and his associates (3) have explained some of the features of the syndrome, including the diabetes, by the action of these steroids, and other features by the action of other adrenal steroids. Evidence in support of these views has been found in an increased excretion of carbohydrate-active material in the urine as measured by stimulation of glycogenesis in the liver of the adrenalectomized animal (4), and also as measured by less specific chemical methods (5). It was therefore a matter of considerable interest to attempt to isolate the active material from the urine when a case of Cushing’s syndrome presented itself in which the associated diabetes mellitus was unusually severe. In this case the excretion of corticosteroid-like substances varied between 14 and 19 mg. (in terms of 11-dehydrocorticosterone) per day. The patient, a boy 14 years of age, was found at surgical exploration, and later at necropsy, to have hyperplastic adrenal cortices.

The method for the determination of corticosteroid-like substances was based on the procedure of Lowenstein, Corcoran, and Page (6), in which the amount of formaldehyde generated by the action of periodic acid is determined. The amount of formaldehyde is taken as a measure of the amount of the groups —COCH₂OH and —CHOHCH₂OH, the former group being an essential characteristic of the cortical hormones. In order to avoid interference by the steroid residues and impurities the formaldehyde was distilled prior to the colorimetric procedure.

The urine was collected daily, acidified to pH 1, and extracted with chloroform after standing 1 to 3 days at room temperature. The extracts were pooled and worked up when enough had been accumulated. It was

1 A report of this case, including metabolic studies, will be presented elsewhere.
decided to use the method of solvent partition that had been used successfully for the isolation of hormones from extracts of the adrenal cortex (7). To this end the chloroform was removed, the residue was taken up in benzene, and the benzene solution was extracted fifteen times with an equal volume of water. The combined aqueous extracts were concentrated to about 250 ml. and extracted with chloroform. Concentration of the chloroform solution to a small volume led to separation of crystals which were filtered out and thoroughly washed with chloroform. A total of 292 mg. of crude crystals was thus obtained from a 25 day collection of urine and this crude material yielded 191 mg. of purified 17-hydroxycorticosterone (I). This amount of purified hormone corresponds to an average of 7.6 mg. per day.

The crude crystals melted at 200–205°. After crystallization from absolute alcohol the melting point was 213–215° and $[\alpha]_D^{20} = +167^\circ \pm 3^\circ$. The melting point was not changed by successive crystallizations from acetone and methanol. These properties, together with the formation of a red 2,4-dinitrophenylhydrazone, the development of a yellow-green fluorescence on treatment with concentrated sulfuric acid, and the prompt
reduction of alkaline silver in the cold, suggested the likelihood that the substance was 17-hydroxycorticosterone (8-10). Examination of the absorption in the ultraviolet with a Beckman spectrophotometer revealed an absorption maximum at 242 m\(\mu\) (\(\epsilon = 15,800\)) which is characteristic of an \(\alpha,\beta\)-unsaturated ketone group. The structure of the compound was firmly established by oxidation to adrenosterone (II) (8, 9) and by oxidation of the acetate (III) to 17-hydroxy-11-dehydrocorticosterone acetate (IV) (10).

The only other structures that would possibly give one or more of these results are Reichstein's (11) Substances E (V) and U (VI). Both have the \(\alpha,\beta\)-unsaturated ketone group and both would yield adrenosterone on oxidation with chromic acid. The former compound, however, melts at 126-127° and \([\alpha]_b^0 = +87°\). The properties of Substance U more nearly agree with those of the urinary compound. It melts at 208° and \([\alpha]_b^{21} = +178.5°\) (acetone). It is excluded from further consideration, however, by the fact that it forms the 20,21-diacetate (m.p. 252-253°) under the conditions used for acetylation of the urinary compound. This diacetate would not be susceptible to oxidation to 17-hydroxy-11-dehydrocorticosterone acetate. Indeed, Reichstein and von Euw (11) converted the diacetate of Substance E to the diacetate of Substance U by oxidation with chromic acid. Substances E and U are further excluded by their failure to reduce alkaline silver solution at room temperature.

Dr. D. J. Ingle kindly consented to assay the compound by his muscle-work test and reported that it contained 9.66 units per mg. Dr. Ingle stated that this value is within 15 per cent of the value recently obtained in his laboratory for 17-hydroxycorticosterone.

The isolation of appreciable quantities of 17-hydroxycorticosterone from the urine in this case is in accord with the view that at least some of the manifestations of Cushing's syndrome are primarily the result of an overproduction of those adrenal cortical hormones which are active in carbohydrate metabolism. Although there is no way of estimating the amount of 17-hydroxycorticosterone that was produced by the hyperplastic adrenal cortices, it seems probable that the amount excreted in the urine could not have been more than 10 per cent of the amount produced, and very likely was much less. This estimate is based on the low recovery of corticosteroids in the urine when 11-dehydrocorticosterone and 17-hydroxy 11-dehydrocorticosterone were given to human subjects (12).

The presence in this case of severe diabetes which was refractory to insulin appeared to be analogous to the insulin-resistant hyperglycemia and glycosuria induced by Ingle, Sheppard, Evans, and Kuizenga (13) in rats by administration of 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone.
ExPERIMENTAL

Extraction and Isolation—The urine was collected in periods of 24 hours. The volume of urine excreted during 24 hours was between 3 and 4 liters. After removal of a small aliquot for various determinations the remainder was acidified to pH 1 with concentrated HCl and allowed to stand 1 to 3 days at room temperature. It was then extracted four times with 0.15 volume of chloroform that had been distilled over K₂CO₃. The combined chloroform extracts were concentrated under reduced pressure and at a bath temperature of 50° to 20 to 30 ml. Several such extracts were combined, diluted to 100 to 200 ml. with chloroform, washed three times with 10 ml. of cold 0.1 N NaOH for each 100 ml. of chloroform solution, and then washed three times with a volume of water equal to that of the NaOH. The chloroform was distilled under reduced pressure at a bath temperature of 50°. The residues were pooled in a little wet chloroform and stored in the refrigerator.

The pool of material obtained during a 16 day period was evaporated to dryness under the conditions just described and the residue was extracted with successive portions of redistilled thiophene-free benzene until 100 ml. of benzene had been used. The residue that did not dissolve readily in benzene was dissolved in 5 ml. of alcohol and this solution was added to the benzene solution. The benzene solution was shaken thoroughly fifteen times with 100 ml. portions of water. When 500 ml. of aqueous extract had been accumulated, it was washed once with 25 ml. of benzene which were added to the main benzene solution. This procedure was repeated with the second and third 500 ml. accumulations of aqueous extract as they were obtained. The benzene residue was put aside in the refrigerator for further examination. The combined aqueous extracts were concentrated under reduced pressure in a bath kept at 40° to approximately 250 ml. This solution was extracted three times with 50 ml. of chloroform. The extract was filtered and concentrated under reduced pressure to approximately 3 ml. Crystals soon began to separate. After refrigeration for 2 hours the mixture was filtered and the crystals were thoroughly washed with chloroform, which removed almost all of the color. The crystals weighed 187 mg. and melted at 200-205°. A second pool of extracts covering a period of 9 days was treated similarly and yielded 105 mg. of crystals, which melted at 202-205°.

The crude crystals were recrystallized successively from absolute alcohol, acetone, and methanol. The melting point after crystallization from absolute alcohol was 213-215° and was not changed by recrystallization from acetone and then from methanol. The 292 mg. of crude crystals
yielded 191 mg. of recrystallized material. The material that was recrystallized from methanol was prepared for analysis.

C_{27}H_{30}O_{5}. Calculated, C 69.57, H 8.35; found, C 69.81, H 8.49

A few crystals of the isolated material developed a yellow-green fluorescence when treated with a drop of concentrated sulfuric acid. A red dinitrophenylhydrazone precipitated when a small amount of material in 0.5 ml. of alcohol was treated with 0.5 ml. of a solution of 2,4-dinitrophenylhydrazine in 2 N HCl (Brady’s reagent).

For determination of the specific rotation 14.7 mg. of substance were dissolved in 5.0 ml. of 95 per cent alcohol: \([\alpha]_{D}^{20} = +167^\circ \pm 3^\circ\). Reichstein (9) observed \([\alpha]_{D}^{20} = +167.2^\circ \pm 2^\circ\) \((e = 1.029 \text{ in absolute alcohol})\).

For examination of the ultraviolet absorption 1.253 mg. were dissolved in 95 per cent alcohol and the solution was made up to 100 ml. Measurements were made with a Beckman spectrophotometer. An absorption maximum was observed at 242 mp; \(E_{1 \text{cm}} = 0.348\) and \(e = 15,800\) \((\log e = 4.2)\).

Preparation of Acetate—A solution of 24.6 mg. of material in 10 drops of pyridine and 3 drops of acetic anhydride was allowed to stand 24 hours at room temperature. Water and HCl were then added and the precipitate was collected on a filter and washed with water. It was dissolved in hot methanol, the solution was filtered, concentrated to about 1 ml., and cooled. The first crop of crystals melted at 218–219°. Recrystallization from acetone did not change the melting point. Combination with the second and third crops from the first methanol solution and recrystallization from this solvent gave 19 mg. of large crystals which melted at 219–220°.

C_{27}H_{29}O_{6}. Calculated, C 68.29, H 7.99; found, C 68.09, H 8.32

Oxidation of 17-Hydroxycorticosterone to Adrenosterone—A solution of 15.0 mg. \((0.0414 \text{ mm})\) of the steroid in 1.5 ml. of glacial acetic acid was treated with 0.55 ml. of 0.902 N chromic acid in 90 per cent acetic acid \((12 \times 0.0414 \text{ milliequivalent})\). Addition of the chromic acid gave a brown, amorphous precipitate which slowly disappeared on standing. After 20 hours a few drops of methanol were added to destroy any excess chromic acid; then water was added, and the mixture was extracted three times with ethyl acetate. The extract was washed with sodium carbonate solution and water, dried over Na_{2}SO_{4}, and evaporated to dryness. The residue was taken up in a little methanol and dry ether was added. A gelatinous precipitate was centrifuged out and discarded. The solution was evaporated to dryness and the residue was crystallized from dry ether. The first crop weighed 3.6 mg. and melted at 217–219°. A mixture with
an authentic specimen of adrenosterone (m.p. 218–220°) melted at 217–219°. In 95 per cent alcohol \([\alpha]_D^{20} = +270° \pm 8° (c = 0.1183)\). Reichstein (14) observed for adrenosterone (Substance G) \([\alpha]_D = +262°\).

Oxidation of 17-Hydroxycorticosterone Acetate to 17-Hydroxy-11-dehydrocorticosterone Acetate—The acetate (9 mg., 0.0223 mm) was dissolved in 0.5 ml. of acetic acid, and 0.0669 milliequivalent of chromic acid in 90 per cent acetic acid was added. After 22 hours at room temperature water was added and the mixture was extracted with ethyl acetate. The extract was washed with sodium carbonate solution and water, dried, and evaporated. The residue was crystallized from acetone and gave two crops of 4.0 and 2.2 mg. which melted at 239–240° and 238–240°, respectively. A mixture of the first crop with an authentic specimen of 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 239–240°) melted at 239–240°.

SUMMARY

17-Hydroxycorticosterone has been isolated from the urine in a case of Cushing’s syndrome associated with severe diabetes mellitus. From a 25 day collection of urine 191 mg. of purified hormone were obtained.

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