HYDROLYSIS OF CONJUGATES OF URINARY CORTICOIDS
WITH β-GLUCURONIDASE

II. THE ISOLATION AND DETERMINATION OF
TETRAHYDROCORTISONE*

BY BILLY BAGGETT,† RALPH A. KINSSELLA, JR.,‡ AND EDWARD A. DOISY

(From the Departments of Biological Chemistry and Internal Medicine, St. Louis University School of Medicine, St. Louis, Missouri)

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Hydrolysis of conjugates of urinary neutral reducing lipides by means of
β-glucuronidase of bacterial origin, as described in Paper I of this series
(1), permits the extraction from urine of much larger amounts of these sub-
stances than may be obtained by extraction of acidified urine in separatory
funnels. The isolation of a crystalline reducing steroid from normal urine
which had been treated with β-glucuronidase was reported at the meeting
of the American Society of Biological Chemists in Cleveland in 1951; this
steroid subsequently was found to be identical with pregnane-3α,17α,21-
triol-11,20-dione (tetrahydrocortisone). The isolation and identification
of this compound from normal urine were also reported by Schneider (3)
at the same meeting. Previously, Lieberman, Hariton, and Dobriner (4)
had reported its isolation from urinary extracts which had been incubated
with β-glucuronidase from calf spleen. The urines were obtained from
subjects who had been treated with ACTH (adrenocorticotropic) and corti-
sone acetate.

Several formaldehydogenic and reducing lipides had been isolated from
normal urine prior to the full appreciation of the desirability of hydrolyzing
conjugated products of excretion. These were 17-hydroxy-11-dehydro-
corticosterone (cortisone) (5), 17-hydroxyxycorticosterone (Kendall’s Com-
 pound F) (6), pregnane-17α,21-diol-3,11,20-trione (6), and tetrahydro-
cortisone (5), each of which was found in a small amount, less than 100 γ
per liter of normal urine. Subsequently, Schneider reported the isolation

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† The material presented herein is taken, in part, from a thesis submitted to the
Graduate School of St. Louis University by Billy Baggett in partial fulfilment of
the requirements for the degree of Doctor of Philosophy in Biochemistry. Present
address, Massachusetts General Hospital, Boston 14, Massachusetts.

‡ John and Mary R. Markle Scholar in the Medical Sciences.

† Oral presentation of data not included in the printed abstract (2).
HYDROLYSIS OF CONJUGATES. II

of tetrahydrocortisone (214 μg per liter) from normal urine which had been incubated with β-glucuronidase prepared from calf spleen (3, 6). In studies of urines obtained from patients with Cushing's syndrome and from patients treated with ACTH, Mason and Sprague (7) and Mason (8) isolated Kendall's Compound F; following treatment with cortisone, this compound was obtained unchanged from the urine by Mason. Other important contributions have been made to the following aspects of the corticoid field: the hydrolysis of conjugates by the continuous extraction of acidified urine by Pincus and Romanoff (9), evidence for occurrence of several conjugates of adrenal cortical metabolites in urine presented by Marrian and Cox (10, 11), and the introduction of paper chromatography to the study of corticoids present in urine by Burton, Zaffaroni, and Keutmann (12).

Since the urinary neutral reducing lipides and formaldehydogenic lipides, measured after enzymatic hydrolysis, have been related to adrenal cortical function (1, 2), the study of a major component of this fraction should yield valuable information concerning the metabolism of adrenal cortical steroids. This paper is a report on the isolation of tetrahydrocortisone from normal urine which had been incubated with β-glucuronidase and on the daily excretion of tetrahydrocortisone by a number of subjects under normal and experimental conditions.

EXPERIMENTAL

Isolation of Tetrahydrocortisone from Pooled Urine—Preliminary studies with paper chromatography as described below indicated marked increases in two reducing compounds following incubation of human urine with bacterial β-glucuronidase. One of these compounds was isolated and identified as tetrahydrocortisone by the following procedure; the other has not yet been identified but the evidence now available indicates that it is not a steroid.

31 liters of pooled urine collected from normal males were subjected to hydrolysis with bacterial β-glucuronidase. Approximately 50,000 units were utilized per liter of urine, and the incubation was carried out for 48 hours at pH 6.2 and at a temperature of 37°.

Following incubation, the urine was acidified to pH 1 and extracted three times with 0.1 volume of chloroform. The extract of the urine was washed three times with 0.1 volume of 0.1 N NaOH, twice with water, dried with anhydrous sodium sulfate, and evaporated to dryness in vacuo.

The crude residue was dissolved in benzene, and the benzene solution was extracted four times with 2.5 volumes of water. The aqueous solution was extracted three times with equal volumes of chloroform, and the chloroform solution was dried and evaporated in vacuo. The resulting residue
was subjected to a Girard separation with fractional hydrolysis according to the method of Mason (8). The fractionation was repeated on the non-ketonic fraction, the resulting ketonic fractions being combined with those from the original Girard separation.

The ketonic fraction, of which the Girard complex was hydrolyzed by acidification to the dark blue color of Congo red, contained the compound subsequently identified as tetrahydrocortisone. This fraction was chromatographed on magnesium silicate-Celite (1:1). The fractions eluted with mixtures of ether and ethyl acetate were combined and crystallized from a mixture of ethyl acetate and petroleum ether. Repeated crystallizations from this solvent system and from a mixture of benzene and acetone resulted in a crystalline product weighing 24.3 mg. Its melting point of 180–183° was not depressed by admixture with tetrahydrocortisone (m.p. 180–183°) which had been prepared from an authentic sample of its diacetate, kindly furnished by Dr. L. H. Sarett of Merck and Company, Inc. The two samples exhibited the same absorption spectrum when treated with concentrated H₂SO₄ according to the method of Zaffaroni (13). The synthetic compound and the substance isolated from urine were identical with respect to the rate of movement on paper chromatograms.

The results of an elemental analysis carried out on a sample of the urinary compound dried at 60° and 1 mm. for 8 hours indicated a monohydrate of tetrahydrocortisone. These results were as follows:

\[\text{C}_{31}\text{H}_{32}\text{O}_{6} \cdot \text{H}_2\text{O}.\] Calculated, C 65.94, H 8.96; found, C 65.20, H 9.04

A small quantity of impure material obtained from the mother liquors in the crystallization of the urinary compound was acetylated with acetic anhydride in pyridine. 3 mg. of crystalline material melting constantly at 225–228° were obtained from the reaction mixture. It did not depress the melting point of an authentic specimen of tetrahydrocortisone diacetate which also melted at 225–228°. Other workers have reported melting points of 233–236° (14), 234–238° (4), and 226–228° (5).

Although the total quantity of tetrahydrocortisone isolated from the 31 liters of urine was 26.7 mg. (0.86 mg. per liter), subsequent studies of the method of hydrolysis and extraction carried out on the same batch of urine clearly showed that an inadequate amount of enzyme for hydrolysis and

2 All melting points reported here were determined on a Fisher-Johns apparatus and are uncorrected. The melting points of both tetrahydrocortisone and its diacetate varied markedly with the degree of pulverization, a phenomenon reported by other workers as occurring with certain other steroids of this type. The melting points given were obtained on samples pulverized as finely as possible.

3 The rate of movement of the compound isolated from urine corresponded to the more mobile of the two principal spots observed on paper chromatograms of urinary extracts. See "Discussion" in a later section of this paper.
an insufficient quantity of organic solvent for the extraction had been used. On the basis of these later experiments in which adequate amounts of enzyme and more efficient extraction were used in combination with paper chromatography, it was found that somewhat less than half of the tetrahydrocortisone had been obtained from the urine used in the isolation work. These results were so interesting that it seemed desirable to ascertain values for the excretion of tetrahydrocortisone for a number of normal individuals.

Preparation of Urinary Extracts for Chromatography—24 hour urine samples were collected, preserved with chloroform, and stored at 10°. 40 to 80 ml. aliquots were incubated for 24 hours at pH 6.2 and 37° with 0.05 volume of bacterial β-glucuronidase (from 1500 to 2000 units per ml.) prepared according to the method of Buehler et al. (15). At the end of this period, the same volume of enzyme was again added and the incubation carried out for another 24 hours. During the incubation, the urine was preserved with a few drops of chloroform to prevent the growth of microorganisms.

At the end of the incubation period, the urine was acidified to pH 1 with 4 M H₂SO₄ and extracted four times with equal volumes of a mixture of 4 parts of redistilled ether (Mallinkrodt, U. S. P., concentrated) and 1 part of redistilled chloroform (Mallinkrodt, analytical reagent). This method of extraction, which is similar to that of Heard et al. (16), gave emulsions only rarely. The ether-chloroform extract was washed three times with 0.05 volume of chilled 0.1 N NaOH and three times with a similar volume of water. It was then dried with anhydrous sodium sulfate, evaporated to dryness in vacuo at a bath temperature below 40°, and dissolved in 1.0 ml. of absolute ethanol. This extraction procedure resulted in recoveries of from 90 to 95 per cent of the tetrahydrocortisone from aqueous solutions.

Paper Chromatography—These extracts were subjected to paper chromatography in the propylene glycol-toluene system described by Burton, Zaffaroni, and Keutmann (17). The samples were introduced onto the propylene glycol-saturated strips in 0.25 ml. of the solution in absolute ethanol (equivalent to 10 to 20 ml. of urine). This volume was measured by counting the number of drops of solution delivered from a 24 gage hypodermic needle which had been calibrated previously. Each drop was allowed to evaporate before the next was released; the rate of evaporation was increased by directing a stream of nitrogen against the underside of the strip which was suspended between two inverted beakers. This method resulted in a spot approximately 1 cm. in diameter. Triplicate strips were prepared for each urinary extract.

A series of known steroids was chromatographed with each set of urinary extracts to serve as standards for the identification of the steroids on the chromatograms of urinary extracts. Those used routinely were tetrahydrocortisone and Kendall’s Compounds E and F.
The chromatograms were allowed to develop from 5 to 8 days, depending upon the rate of movement of tetrahydrocortisone as determined by occasionally developed strips. None of the spots found on chromatograms after this period could be resolved into additional spots by longer periods of development. The developed chromatograms were removed from the chamber and allowed to dry at room temperature, and then one of each set of triplicates was treated with ammoniacal silver hydroxide according to the method of Burton, Zaffaroni, and Keutmann (17). These strips served to locate the α-ketolic steroids and to approximate the amount of each present. The other paper strips from each set were cut into sections corresponding to spots observed on the first. Completeness of recovery of the tetrahydrocortisone spot was checked by developing the cut ends. Each section was elutriated with three 3 ml. washings of methanol and filtered through a sintered glass funnel. The elutriates were taken to dryness under a stream of nitrogen. Corresponding sections from blank strips of paper were extracted in the same manner to serve as blanks in the quantitative determination.

Determination of Tetrahydrocortisone—Since the principal urinary α-ketolic steroids were found to be 17,21-dihydroxy-20-ketosteroids, the phenylhydrazine method of Porter and Silber (18) was utilized for the quantitative measurement of these compounds in elutriates from the chromatograms. Control experiments with this reaction showed that the intensity of the color is proportional to the amount of tetrahydrocortisone and that the color is stable for at least 2½ hours. Readings were made at the wavelength, 415 μ, at which maximal absorption occurred.

The values reported in this paper were obtained from the readings of the colored products resulting from the application of this reaction to the residues of elutriates from the paper strips after correction for the color contributed by the paper blanks. In the earlier phases of this work, the paper blanks were found to be quite variable from analysis to analysis, but were more constant within an individual experiment. The average amount of color produced by the blanks, representing 6 to 12 cm. of paper strip, was equivalent to that given by from 10 to 20 γ of tetrahydrocortisone.

In order to improve the accuracy of the quantitative method, another step was introduced which reduced the blanks to values too small to be measured. The residues obtained after evaporation of the methanol used for elutriation were transferred with 25 ml. of chloroform to a separatory funnel. The chloroform was shaken with 5 ml. of water and separated. The water was shaken with 5 ml. of chloroform and the chloroform portions were combined. The chloroform was removed under a stream of nitrogen, a hot water bath to facilitate the evaporation being employed. This procedure, which removes 96 per cent of the propylene glycol originally present in the chloroform, reduces color formation of the blanks to negligible
amounts. The recovery of tetrahydrocortisone added to the chloroform and subjected to the procedure outlined above amounted to 96.5 per cent.

The failure to obtain values for the recovery of tetrahydrocortisone added to urine more closely approaching 100 per cent is due to two factors: (1) the necessity of washing the initial extract of urine, and (2) the impossibility of complete recovery from the paper chromatogram.

The first factor was examined by determining the partition ratio of tetrahydrocortisone between water and the mixture of ether-chloroform (4:1). On the basis of the partition ratio, it was calculated that 8 per cent of the steroid would be removed from the organic phase by the washings used to prepare the extract for chromatography. In an experimental study of this point, 92.5 per cent of the steroid remained in the organic phase.

In a study of the second factor, the recovery of tetrahydrocortisone from paper strips was studied. This included extraction with methanol and the washing of the chloroform solution of the elutriate with water to reduce the value of the blank to zero. The recovery approximated 90 per cent, values ranging from 88.1 to 91.6 per cent.

If these demonstrated and evaluated losses (8 and 10 per cent) are taken into account, it is apparent that values no larger than 82 per cent of the amount of tetrahydrocortisone present in urine should be obtained. In practice, the results of the over-all recovery of tetrahydrocortisone have ranged from 73 to 84 per cent with an average close to 80 per cent. Accordingly, in view of these measured losses, it is apparent that the values reported in Tables I and II are only 80 per cent of the amounts actually present in urine.

The daily urinary excretion of tetrahydrocortisone by several individuals is given in Table I. These values range from 2.0 to 7.4 mg. per 24 hours. The excretion of Subject 1 is of interest because it is much larger than those of the other male subjects and the values (5.1 to 7.4 mg. per 24 hours) are remarkably constant. After the administration of ACTH to Subject 1, the amount of tetrahydrocortisone excreted in the 24 hour urine was increased to approximately 3 times the average of the control values (Table II). This, coupled with the recovery in the urine of tetrahydrocortisone equivalent to 28 per cent of the cortisone which had been taken orally by Subject 1 and to 18.5 per cent of the cortisone which had been taken orally by Subject 2, indicates that cortisone is a precursor of tetrahydrocortisone and that the adrenal secretes cortisone or a substance similar in character which is metabolized to tetrahydrocortisone. The low value for tetrahydrocortisone in the urine obtained from a patient with panhypopituitar-

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4 In some of these experiments, the reducing power of tetrahydrocortisone (1) was utilized for estimation. The reducing power of 1.0 mg. of desoxycorticosterone is equivalent to that of 2.2 mg. of tetrahydrocortisone.
ism (Sheehan’s syndrome) and the elevated figure obtained on urine from a
patient with Cushing’s syndrome also indicate that the excretion of this
compound is related to adrenal cortical function. The recovery of in-
creased urinary tetrahydrocortisone after the administration of Compound
F indicates that it also may be a significant precursor of tetrahydrocortisone
(Table II).

**Table I**

**Urinary Excretion of Tetrahydrocortisone**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Description</th>
<th>Volume of 24 hr. urine</th>
<th>Tetrahydrocortisone per 24 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal male</td>
<td>2640</td>
<td>5.5</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>2310</td>
<td>6.7</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>2880</td>
<td>6.0</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>2020</td>
<td>5.7</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>2500</td>
<td>5.1</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>3380</td>
<td>5.8</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>2480</td>
<td>5.3</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>2180</td>
<td>7.4</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>580</td>
<td>2.0</td>
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<td>580</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>780</td>
<td>2.7*</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3540</td>
<td>3.9</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>930</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>910</td>
<td>3.5</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>710</td>
<td>3.3</td>
</tr>
<tr>
<td>7</td>
<td>Pregnant female</td>
<td>2330</td>
<td>2.2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>2275</td>
<td>2.4</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>2290</td>
<td>2.2</td>
</tr>
<tr>
<td>9</td>
<td>Female, panhypopituitarism</td>
<td>410</td>
<td>0.4</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>1125</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>Male, Cushing’s syndrome</td>
<td>1780</td>
<td>12.1</td>
</tr>
</tbody>
</table>

* The subject was suffering from a mild infection of the upper respiratory tract.

**Other Compounds Detected on Paper Chromatograms**—In addition to tetra-
hydrocortisone, three other compounds which reduced ammoniacal silver
were observed on the chromatograms obtained from the extracts of urine
which had been hydrolyzed with β-glucuronidase. Two of these corre-
sponding to Kendall’s Compounds E and F were present at a concentration
of from 100 to 200 γ per 24 hour urine sample, a 3- to 4-fold increase over
the quantity obtained from unhydrolyzed urine. The third compound and
tetrahydrocortisone were less mobile than Compounds E and F. They
were present in much greater concentrations, totaling approximately 1.0 to
8.0 mg. per 24 hour urine as estimated by the intensity of the spots result-
ing when the chromatograms were treated with the ammoniacal silver
reagent. This represented a 10- to 50-fold increase over their concentrations in extracts of unhydrolyzed urine.

The more mobile of these two compounds was demonstrated to be tetrahydrocortisone by isolation and identification as outlined above. The less mobile compound has not yet been identified. This compound does not react with the phenylhydrazine reagent of Porter and Silber to form the

**Table II**

*Urinary Tetrahydrocortisone after Administration of ACTH, Compound E Acetate, and Compound F*

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Material administered</th>
<th>Route of administration</th>
<th>Excretion of tetrahydrocortisone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mg. per 24 hrs.</td>
</tr>
<tr>
<td>1</td>
<td>120 units ACTH</td>
<td>Intramuscular</td>
<td>5.9</td>
</tr>
<tr>
<td>1</td>
<td>400 mg. Compound E acetate</td>
<td>Oral</td>
<td>5.9</td>
</tr>
<tr>
<td>1</td>
<td>200 &quot; &quot; &quot; &quot; F</td>
<td>Intramuscular</td>
<td>5.9</td>
</tr>
<tr>
<td>1</td>
<td>400 &quot; &quot; &quot; F</td>
<td>Oral</td>
<td>5.9</td>
</tr>
<tr>
<td>1</td>
<td>200 &quot; &quot; &quot; &quot;</td>
<td>Intramuscular</td>
<td>5.9</td>
</tr>
<tr>
<td>2</td>
<td>385 &quot; &quot; &quot; E acetate</td>
<td>Oral</td>
<td>2.2</td>
</tr>
<tr>
<td>2</td>
<td>400 &quot; &quot; &quot; F</td>
<td>&quot;</td>
<td>2.2</td>
</tr>
</tbody>
</table>

The subjects were normal males identified as in Table I. Analyses were carried out simultaneously with those on control urines (Table I). Three intramuscular injections of 40 units of ACTH each were given at intervals of 4 to 6 hours. Steroids were administered in three equally divided portions at intervals of 4 hours. The collection of urine covered the 24 hour period beginning with the administration of the first dose. The heading of the last column, per cent conversion, is used for convenience. Although the increased excretion is due to the administered compound, and we believe that the compound is converted to tetrahydrocortisone, we have no absolute proof of this conversion. ACTH (corticotropin, Wilson) was furnished by Dr. David Klein, The Wilson Laboratories, Chicago, Illinois. Adrenal steroids, Compound E acetate, and Compound F were supplied by Dr. Elmer Alpert, Merck and Company, Inc., Rahway, New Jersey.

characteristic yellow color, thereby indicating the absence of a 17,21-dihydroxy-20-keto grouping.\(^5\) A reducing compound of similar mobility is also found in ether-chloroform extracts of the *Escherichia coli* culture medium used as a source of glucuronidase. The culture fluid, however, was not the source of this compound appearing on chromatograms of urinary extracts, since it still appeared after the culture fluid had been exhaustively extracted with the ether-chloroform mixture before being used for hy-

\(^5\) Since the unidentified compound observed on the chromatograms fails to give the characteristic colored products with phenylhydrazine in sulfuric acid, it is improbable that it is pregnane-3α,11β,17α,21-tetrol-20-one.
drolysis of urinary conjugates. Chromatograms of extracts of incubated enzyme which had previously been extracted showed no areas which reduced ammoniacal silver hydroxide. The quantity of this reducing compound in the urine was increased by the administration of adrenocorticotropic, Kendall's Compound F, and cortisone. Its concentration in all urines studied was approximately one-half that of tetrahydrocortisone, as judged by the intensities of the reduced silver spots on the chromatograms.

**DISCUSSION**

Tetrahydrocortisone was first isolated from normal human urine by Schneider, who obtained 6 mg. as the diacetate from 1000 liters of unhydrolyzed urine (5). Lieberman, Hariton, and Dobriner, using β-glucuronidase prepared from calf spleen, isolated an unspecified quantity from extracts prepared from the urine of subjects receiving adrenocorticotropic or cortisone (4). The importance of this compound as an adrenal cortical metabolite was subsequently suggested by Schneider, who isolated 18.5 mg. as the diacetate from 70 liters of urine (3, 5) which had been incubated with β-glucuronidase, by Baggett, Glick, and Kinsella (19), and by Dohan and Richardson (20). No other α-ketolic steroid has been found on paper chromatograms of urinary extracts in quantities approaching the quantity of tetrahydrocortisone found in this study.

Although pregnane-3α, 11β, 17α, 21-tetrol-20-one (tetrahydro F) has been isolated by Lieberman, Hariton, Stokem, Studer, and Dobriner from urine collected from patients receiving adrenocorticotropic (21), it has not been encountered in our work either on paper chromatograms or during the isolation of tetrahydrocortisone. Unfortunately, this compound has not been available to us for a study of its movement on paper chromatograms, but it seems likely that it would be separated from tetrahydrocortisone since other compounds differing only in the nature of the 11-oxygen function are well separated. Zaffaroni and Burton were able to separate allopregnane-3β, 17α, 21-triol-11, 20-dione from allopregnane-3β, 11β, 17α, 21-tetrol-20-one by this system of paper chromatography (22). In the isolation work reported in this paper, the quantity of tetrahydrocortisone obtained was about 90 per cent of that estimated to be present in the crude extract by means of paper chromatography. This offers further evidence that the single spot assigned to tetrahydrocortisone is not actually a mixture containing appreciable quantities of any other α-ketolic steroid. While the available evidence indicates that quantitatively the 11-hydroxy compound is of minor importance in normal urine as compared with that of the 11-keto compound, tetrahydrocortisone, the possibility of excretion of the 11-hydroxy compound in a conjugated form which has not yet been successfully hydrolyzed does exist.

The chemical structure of tetrahydrocortisone clearly indicates that it is
a metabolite of an adrenal cortical steroid or steroids. The marked increase in the excretion of this compound following the administration of adrenocorticotropin, Compound E, and Compound F affords more evidence that tetrahydrocortisone is a metabolite of compounds formed in the adrenal.

SUMMARY

By use of the paper chromatographic method of Zaffaroni, tetrahydrocortisone has been found to be the principal \( \alpha \)-ketolic steroid extractable from human urine after hydrolysis with bacterial \( \beta \)-glucuronidase. The compound was identified by isolation in crystalline form and characterization. The daily excretion of this compound by the group of normal individuals studied was 2.0 to 7.4 mg. The excretion of tetrahydrocortisone was markedly increased by the administration of adrenocorticotropin and Kendall’s Compounds E and F.

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