THE CHARACTERIZATION OF FOUR NEW METABOLITES OF ADRENOCORTICAL HORMONES*

By DAVID K. FUKUSHIMA, NORMA S. LEEDS, H. LEON BRADLOW, THEODORE H. KRITCHEVSKY,† MADELEINE B. STOKEM, AND T. F. GALLAGHER

(From the Sloan-Kettering Institute for Cancer Research, New York, New York)

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This report describes the isolation and characterization by partial synthesis of four metabolites of the adrenal hormones. These products (Fig. 1) are pregnane-3α,11β,17α,20α,21-pentol (I), for which we propose the trivial name "cortol;" 3α,17α,20α,21-tetrahydroxypregnane-11-one (II), proposed trivial name "cortolone;" pregnane-3α,11β,17α,20β,21-pentol (III), proposed trivial name "β-cortol;" 3α,17α,20β,21-tetrahydroxypregnane-11-one (IV), proposed trivial name "β-cortolone."[1]

All four compounds have been isolated from human urine after the administration of corticotropin (ACTH) for therapeutic purposes. By the method of isotopic dilution they have been found and measured in human urine after a tracer dose of hydrocortisone-4-C^{14}. Cortolone was likewise found in the urine after administration of 100 mg. of cortisone-l[2] to a human subject. There is little doubt that these four metabolites are normal constituents of human urine, and studies of this aspect will be reported in the near future.

By analogy with other 20-hydroxy steroids isolated from human urine, it was anticipated that the 20α epimer would be the predominant metabo-

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† Present address, Ninol Laboratories, 1719 South Clinton Street, Chicago, Illinois.

[1] These names are suggested in order to avoid perpetuation of the "tetrahydro E" type of nomenclature. It is emphasized that there is no intention to construct a system based upon these trivial designations. For this reason the indefinite "β-cortol" is recommended rather than the more specific "20β-cortol," since the precise usage might lead to further extension, *e.g.*, "3β,20β-cortol," etc. It is strongly urged that the use of these names be limited to the compounds described and that other epimers, which will undoubtedly be discovered, receive appropriate new trivial names.

[2] Radioactive cortisone, with 95 per cent of the tritium label in known, stable positions in the molecule (*cf.* Fukushima *et al.* (1)).

lite. Accordingly, efforts were directed primarily toward an efficient, readily applicable synthesis for cortolone (II), since this was the least accessible compound by known methods. II was first obtained by Sarett (2) in 10 per cent yield, together with 65 per cent of the 20β epimer from the catalytic reduction of 3α,17α,21-trihydroxypregnane-11,20-dione. Soloway, Considine, Fukushima, and Gallagher (3) have reported the synthesis of II from 3α,21-diacetoxy-$\Delta^{17(20)}$-pregnene-11-one, but the relative unavailability of the starting material limited the use of this procedure. The method that has been devised starts from the readily accessible 3α,21-diacetoxy-17α,20β-dihydroxypregnane-11-one (2) which was converted to the 20-tosylate, followed by acetylation of the 17α-hydroxyl group. When the triacetoxy tosylate (VI) was heated with potassium acetate in aqueous acetic acid, acetyl migration with inversion occurred and cortolone triacetate (VII) was obtained in high yield.

EXPERIMENTAL[3]

Sources of Urine and Methods of Fractionation. (*a*) *ACTH*—A 26 day urine collection was obtained from a female patient with Hodgkin's disease while she received 100 mg. of ACTH intramuscularly daily. The urine was acidified to pH 1 and extracted continuously with ether for 48 hours. The neutral fraction was separated into ketonic and non-ketonic fractions, as previously described (4). The non-ketonic fraction was partitioned between water and benzene by the procedure of Mason (5). The water-soluble extract was acetylated with acetic anhydride and pyridine at room temperature and was further fractionated by partition chromatography (6).

(*b*) *Hydrocortisone*—Hydrocortisone-4-C^{14} (about 0.25 mg.; 0.855 μc.) was administered intravenously during 30 minutes. For this study 61 per cent of the urine (or a total of 117,000 c.p.m.) from Subject F1 (7) obtained during the 2nd hour following the initiation of the infusion was treated with 300 Fishman units per ml. of beef liver β-glucuronidase for 5 days at pH 5. The urine was then extracted continuously with ether for 48 hours, and the neutral ether-soluble fraction was obtained. This crude neutral fraction contained 84,000 c.p.m., equivalent to 72 per cent hydrolysis of the total conjugates present in the urine. Carrier cortolone triacetate (VII, 35.8 mg.) and cortol triacetate (I, 3,20,21-triacetate, 29.4 mg.) were added, and the mixture was acetylated with 10 ml. of acetic anhydride and 5 ml. of pyridine at room temperature overnight. The

[3] The phrase "in the usual manner" indicates that the product was extracted with ether, washed with either acid or base, or both, as appropriate, and then with dilute brine. After drying over sodium sulfate, the solvent was distilled. Unless otherwise noted, rotations were measured in chloroform. The melting points are corrected.

acetylated extract was separated on a partition type chromatogram with 40 per cent ethanol on silica gel as the stationary phase and 1 per cent ethanol in 1:1 methylene chloride-petroleum ether as the mobile phase.

Radioactivity was measured in windowless gas flow counters, and the results were corrected to the "infinitely thin" range.

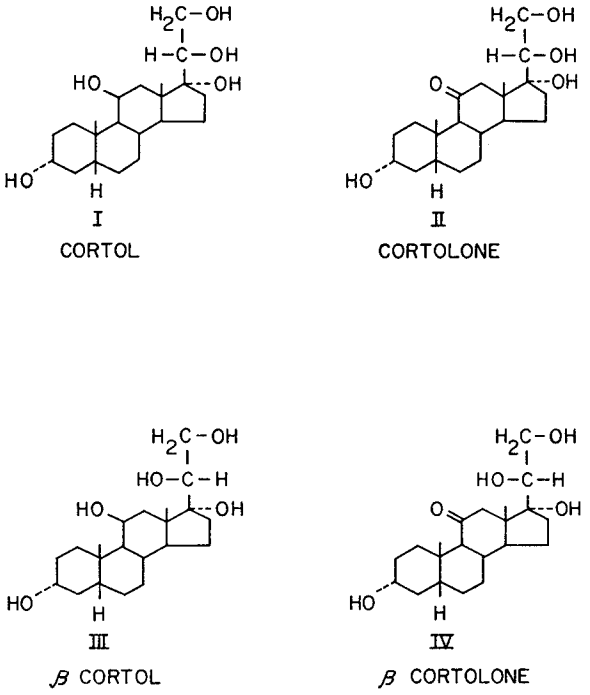

FIG. 1

Cortolone Triacetate (VII). (*a*) *After ACTH Administration*—Several crystalline fractions, eluted from the chromatogram immediately before cortol triacetate, were cortolone triacetate as judged by infra-red spectrometry. These were combined (9 mg.) and recrystallized twice from methanol to yield 4 mg., m.p. 214.5–216°. The product was indistinguishable from an authentic sample by infra-red spectrometry and mixture melting point.

(*b*) *Isolation by Isotopic Dilution after Hydrocortisone-4-C*^{14} *Administration*—After chromatography, the fractions that contained cortolone triacetate as judged by infra-red spectrometry were combined and rechromatographed on a similar column. The purified cortolone triacetate was triturated with cyclohexane and yielded 23.8 mg.; 130 c.p.m. per mg.

The product was recrystallized first from benzene, then from methanol, and again twice from benzene to yield a substance, m.p. 215.5–216.5°; 153 c.p.m. per mg. Two further recrystallizations from methanol and from benzene gave products with 161 and 158 c.p.m. per mg. respectively. The products of the last three crystallizations were assumed to have constant specific activity; from the average of 157 c.p.m. per mg. and the weight of carrier added (35.8 mg.) a minimum of 6.7 per cent of the total radioactivity in the crude neutral fraction was present as cortolone.

(*c*) *Isolation from Urine after Cortisone-t Administration*—The urine from a single day after the intravenous administration of 100 mg. of cortisone-*t* to a female patient was processed as described for the hydrocortisone-4-C^{14} studies. Chromatography of the acetylated crude neutral fraction without the addition of carrier steroid resulted in the isolation of cortolone triacetate, identified by melting point and by infra-red spectrometry. From the total radioactivity in the crude neutral extract (1.08×10^6 c.p.m., 61.5 per cent of the dose administered) and the radioactivity (6.0×10^4 c.p.m.) in the fractions of the chromatogram that contained cortolone triacetate as judged by infra-red spectrometry, it was estimated that 5.6 per cent was present as cortolone.

Cortol Triacetate (*I, 3,20,21-Triacetate*). (*a*) *After ACTH Administration*—Several fractions eluted from the partition type chromatogram after cortolone triacetate and before β-cortolone triacetate were essentially pure cortol triacetate as judged by infra-red spectrometry. These were combined as 7 mg. of semicrystalline product. Two recrystallizations from benzene-cyclohexane yielded 4 mg. of cortol triacetate, m.p. 168–170°, indistinguishable from an authentic sample by infra-red spectrometry and mixture melting point.

(*b*) *Isolation by Isotopic Dilution after Hydrocortisone-4-C^{14} Administration*—The fractions containing cortol triacetate as judged by infra-red spectrometry were combined and triturated with cyclohexane. The insoluble crystalline material weighed 14.7 mg.; 475 c.p.m. per mg. The oily mother liquor was crystallized from benzene-cyclohexane, and the two crystalline crops were combined. Two recrystallizations from benzene-cyclohexane gave cortol triacetate, with a specific activity of 495 c.p.m. per mg. Two subsequent recrystallizations from the same solvent yielded cortol triacetate, m.p. 168–170°; 542 and 548 c.p.m. per mg., respectively, for each recrystallization. The products of the last two crystallizations were assumed to have constant specific activity; from the average of 545 c.p.m. per mg. and the weight of carrier added (29.4 mg.) a minimum of 19 per cent of the total radioactivity in the crude neutral fraction was present as cortol.

β-*Cortol* (*III*). (*a*) *After ACTH Administration*—Several amorphous

fractions from the chromatogram were β-cortol triacetate as judged from infra-red spectrometry. These were combined (9 mg.) and hydrolyzed with methanolic potassium hydroxide under reflux. The product, obtained in the usual manner, was recrystallized from methanol-ethyl acetate to yield β-cortol, m.p. 254–261°, indistinguishable from the authentic compound by infra-red spectrometry (potassium bromide disc) and mixture melting point.

(*b*) *Isolation by Isotopic Dilution after Hydrocortisone-4-C*^{14} *Administration*—The crude neutral extract (71,000 c.p.m.) from the urine collected during the 3rd hour after infusion of the tracer dose in Subject F1 (7) was obtained as described above. 39.4 mg. of carrier β-cortol were added, and the mixture was dissolved in methanol. After removal of the solvent, the residue was extracted several times with small portions of acetone. The almost colorless crystalline residue was recrystallized twice from methanol to yield 21 mg. of β-cortol, m.p. 259–264°; 224 c.p.m. per mg. The product was recrystallized from ethyl acetate-methanol; 101 c.p.m. per mg. A fourth recrystallization gave a product, m.p. 261–266°; 94 c.p.m. per mg. The products of the last two crystallizations were assumed to have constant specific activity; from the average of 98 c.p.m. per mg. and the weight of carrier added (39.4 mg.) 5.4 per cent of the radioactivity in the crude neutral fraction was present as β-cortol.

β-Cortolone Triacetate (*IV, 3,20,21-Triacetate*). (*a*) *After ACTH Administration*—The crystalline material (25 mg.) eluted from the chromatogram after cortol triacetate and before β-cortol triacetate was β-cortolone triacetate as judged by infra-red spectrometry. After recrystallization from methanol 12 mg. of β-cortolone triacetate were obtained, m.p. 203–204°, indistinguishable from an authentic sample by infra-red spectrometry and mixture melting point.

(*b*) *Isolation by Isotopic Dilution after Hydrocortisone-4-C*^{14} *Administration*—All the mother liquors from the isotopic dilution of β-cortol were combined (69,000 c.p.m.; calculated 68,500 c.p.m.). 26.27 mg. of β-cortolone were added, and the mixture was acetylated. Partition chromatography yielded 29 mg. (4000 c.p.m.) of β-cortolone triacetate as judged by infra-red spectrometry. Recrystallization from benzene gave 20 mg., m.p. 203.5–204°; 43 c.p.m. per mg. Recrystallization from methanol and from benzene afforded products with 44 and 39 c.p.m. per mg. respectively. The product from the last three recrystallizations was assumed to have constant specific activity; from the average of 42 c.p.m. per mg. and the weight of the carrier added, corrected to the triacetate (35.3 mg.), a minimum of 2.1 per cent of the total radioactivity in the crude neutral fraction was present as β-cortolone.

3α,21-Diacetoxy-17α-hydroxy-20β-p-toluenesulfonoxypregnane-11-one—A solution of 2.31 gm. of 3α,21-diacetoxy-17α,20β-dihydroxypregnane-11-one (V) and 1.94 gm. of *p*-toluenesulfonyl chloride in 5 ml. of pyridine was stored at 0° for 3 days, and 2.95 gm. of crude tosylate were obtained in the usual manner. Recrystallization from methanol yielded pure 3α,21-diacetoxy-17α-hydroxy-20β-*p*-toluenesulfonoxypregnane-11-one, m.p. 148° (decomposition), $[\alpha]_D^{27}$ +60.6°.

$C_{32}H_{44}O_9S$. Calculated, C 63.55, H 7.33; found, C 63.24, H 7.36

3α,17α,21-Triacetoxy-20β-p-toluenesulfonoxypregnane-11-one (*VI*)—A solution of 223 mg. of 3α,21-diacetoxy-17α-hydroxy-20β-*p*-toluenesulfonoxypregnane-11-one, 40 mg. of *p*-toluenesulfonic acid, 4 ml. of acetic acid, and 2 ml. of acetic anhydride was stored for 3 days at room temperature (8). The reaction mixture was diluted with ether and the product was obtained in the usual manner. Recrystallization from benzene-cyclohexane gave 102 mg. of 3α,17α,21-triacetoxy-20β-*p*-toluenesulfonoxypregnane-11-one (VI), m.p. 156–159° (decomposition). The analytical sample melted at 159–159.5° (decomposition), $[\alpha]_D^{25}$ +50.0°.

$C_{34}H_{46}O_{10}S$. Calculated, C 63.13, H 7.17; found, C 63.01, H 7.29

Solvolysis of 3α,17α,21-Triacetoxy-20β-p-toluenesulfonoxypregnane-11-one (*VI*). (*a*) *With Potassium Acetate in Aqueous Acetic Acid to Yield Cortolone Triacetate* (*VII*)—1 gm. of potassium acetate dissolved in 0.5 ml. of acetic anhydride and 10 ml. of 96 per cent aqueous acetic acid were heated under reflux, and 70 mg. of VI were added. The mixture was refluxed for 3 hours and stored overnight at room temperature. The reaction mixture was poured into ice and water, and the product (58 mg.) was isolated in the usual manner. Recrystallization from methanol yielded 30 mg. of cortolone triacetate (VII), m.p. 214–216°, $[\alpha]_D^{28}$ +28°, indistinguishable from an authentic sample by either infra-red spectrum or mixture melting point. Partition chromatography (6) of the mother liquors gave an additional 11 mg. of triacetate VII, m.p. 202–212°.

(*b*) *With Base*—A small sample of 3α,17α,21-triacetoxy-20β-*p*-toluenesulfonoxypregnane-11-one (VI) was dissolved in 5 per cent potassium hydroxide in methanol and stored overnight at room temperature. The crude product, obtained in the usual manner, was acetylated with pyridine and acetic anhydride at room temperature. From the infra-red spectrum, the reaction product was principally 3α,21-diacetoxy-17α,20α-epoxypregnane-11-one (VIII).

Solvolysis of 3α,21-Diacetoxy-17α-hydroxy-20β-p-toluenesulfonoxypregnane-11-one. (*a*) *With Anhydrous Potassium Acetate in Acetic Acid*—When 123 mg. of 3α,21-diacetoxy-17α,hydroxy-20β-*p*-toluenesulfonoxypregnane-

11-one were refluxed for 2 hours with 127 mg. of freshly fused potassium acetate in 10 ml. of glacial acetic acid, the product (98 mg.) was predominantly 3α,20β,21-triacetoxy-17α-hydroxypregnane-11-one (IV, 3,20,21-triacetate), as judged from the infra-red spectrum.

(*b*) *With Base*—A solution of 1.00 gm. of 3α,21-diacetoxy-17α-hydroxy-20β-*p*-toluenesulfonoxypregnane-11-one in 100 ml. of 0.5 N methanolic sodium hydroxide was stored overnight at room temperature. The product, isolated in the usual manner, was acetylated at room temperature with acetic anhydride and pyridine to yield 645 mg. of product. Recrystallization from methanol gave 360 mg. of 3α,21-diacetoxy-17α,20α-epoxypregnane-11-one (VIII), m.p. 172–172.5° $[\alpha]_D^{27}$ +120°. The infra-red spectrum was different from that of the corresponding 17α,20β-epoxide described by Soloway *et al.* (3), and the melting point was depressed on admixture of the two compounds.

$C_{25}H_{36}O_6$. Calculated, C 69.44, H 8.33; found, C 69.54, H 8.39

Pregnane-3α,11β,17α,20α,21-pentol (*Cortol*) (*I*)—A solution of 2.08 gm. of cortolone triacetate (VII) in 80 ml. of anhydrous benzene and 80 ml. of anhydrous ether was added slowly to a suspension of 2 gm. of lithium aluminum hydride in 700 ml. of anhydrous ether. The mixture was refluxed for 4 hours, and the excess reagent was destroyed with ethyl acetate and dilute sulfuric acid. The reduction product was extracted with large volumes of ethyl acetate and isolated in the usual manner. Recrystallization gave 669 mg. of cortol (I), m.p. 247–253°. The analytical sample melted at 250.5–254°; $[\alpha]_D^{26}$ +23.7° (ethanol).

$C_{21}H_{36}O_5$. Calculated, C 68.44, H 9.85; found, C 68.58, H 9.79

Cortol 3,20,21-triacetate was prepared with acetic anhydride and pyridine at room temperature, followed by recrystallization from benzene-cyclohexane; m.p. 168–170°, $[\alpha]_D^{20}$ 0°.

$C_{27}H_{42}O_8$. Calculated, C 65.59, H 8.50; found, C 65.48, H 8.36

Pregnane-3α,11β,17α,20β,21-pentol (*β-Cortol*) (*III*)—A solution of 450 mg. of β-cortolone triacetate (IV, 3,20,21-triacetate) in 50 ml. of anhydrous benzene and 50 ml. of anhydrous ether was slowly added to a suspension of 1 gm. of lithium aluminum hydride in 250 ml. of anhydrous ether. The mixture was refluxed for 2 hours, and 362 mg. of crystalline product, m.p. 239–243°, were obtained in the usual manner. Recrystallization from methanol yielded 176 mg. of β-cortol (III), m.p. 262–264.5°, $[\alpha]_D^{25}$ +33.3° (ethanol); reported (9) m.p. 266–269°. The mother liquors were acetylated in the usual manner and chromatographed on a partition

column with ethanol and petroleum ether-methylene chloride. 120 mg. of amorphous β-cortol triacetate were eluted.

β-Cortol 3,20,21-triacetate was prepared from pure β-cortol with acetic anhydride and pyridine at room temperature, but has not as yet been obtained in crystalline state.

Reference samples of cortolone and β-cortolone were prepared by the method of Sarett (2). The constants of these products were as follows: cortolone (II), m.p. 208–209°, $[\alpha]_D^{28}$ +34.2° (ethanol); reported m.p. 208–209°, $[\alpha]_D^{25}$ +44° (alcohol); cortolone 3,20,21-triacetate (VII), m.p. 214–216°, $[\alpha]_D^{28}$ +28°; reported m.p. 213–214°,$[\alpha]_D$ +18° (acetone); β-cortolone (IV), m.p. 260–261.5°, $[\alpha]_D^{27}$ +40° (ethanol); reported m.p. 263–264°, $[\alpha]_D^{25}$ +37° (alcohol); β-cortolone 3,21-diacetate (V), m.p. 207–208°, $[\alpha]_D^{30}$ +61°; +63° (acetone); reported m.p. 201–203°, $[\alpha]_D$ +67° (acetone); β-cortolone 3,20,21-triacetate, m.p. 205–206°, $[\alpha]_D^{25}$ +87.3°; +95.9° (acetone); reported m.p. 201°, $[\alpha]_D$ +103° (acetone).

DISCUSSION

In this investigation, a new group of metabolites of the principal adrenocortical hormone, hydrocortisone, has been discovered. These substances represented an appreciable fraction of a tracer dose of labeled hormone during the particular time interval studied and therefore almost surely constitute a significant portion of the excretory products normally derived from hydrocortisone in man. Moreover, these same compounds were found in urine after stimulation by ACTH. Quite apart from structural considerations, this is further evidence for the ultimate adrenal origin of these metabolites.

The view is widely held that the major metabolites of adrenal cortical hormones are either reducing ketols of the "tetrahydro E" and "tetrahydro F" type or 17-ketosteroids of the C_{19} series, such as 11-ketoetiocholanolone. This conviction has persisted despite the fact that these substances account for only a small fraction of administered hormone either by direct isolation or by less rigid criteria such as "reducing steroids" or Porter-Silber chromogens. Since the metabolites described in this investigation are not reducing substances and are negative in the Porter-Silber reaction, they would escape detection by either of these measures. In addition, unless particular care is exercised in the fractionation of urine or tissue extracts containing these compounds, an appreciable portion may be lost as a result of water solubility before isolation or measurement. As an example of this fact, both cortolone and β-cortolone have been detected by their characteristic infra-red spectrum in the "phenolic" fraction when urine extracts were fractionated by the routine procedure used in this laboratory. This is, of course, a consequence of solubility in water, since the compounds themselves have no acidic properties.

For a variety of reasons the quantity of these metabolites measured by isotopic dilution is a minimal estimate of that actually present in the urine. First, for convenience, carrier was added as the acetate rather than the free alcohol present in the extract. Second, the carrier steroids were added to the neutral ether-soluble extract rather than to the original solvent used for extraction of the urine. Third, incomplete hydrolysis by beef liver β-glucuronidase may likewise have lowered the yield from that

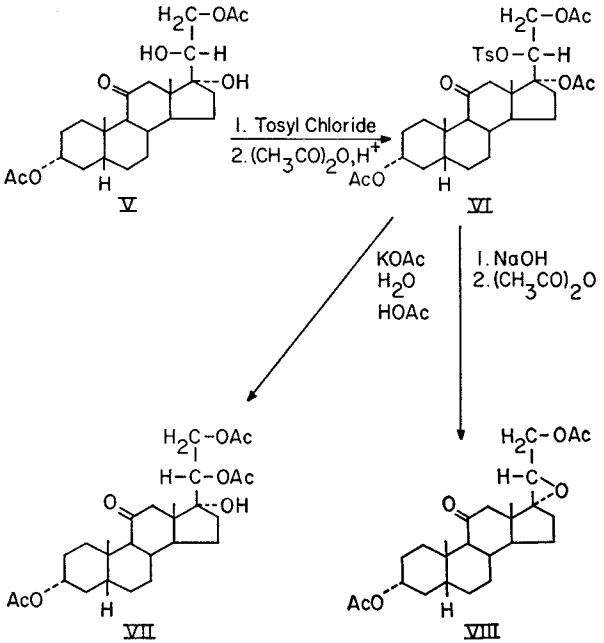

FIG. 2

theoretically attainable. While the amount of urinary radioactivity obtained in the neutral ether-soluble fraction was greater than 70 per cent of the total, it is probable that additional quantities of these metabolites were still conjugated and thus remained either in the extracted urine or in the alkaline extracts. While all of these factors save the hydrolysis are relatively minor, the cumulative effect may be significant and should be recognized for more definitive studies. Similar reservations must be made for the isolation experiments from urine after ACTH administration. Portions of the extracts were used in ancillary studies and the goal with the new compounds was homogeneity rather than yield. The amount of metabolites isolated therefore was but a fraction of the total material present in the original extract.

The reactions involved in the synthesis of cortolone from the readily available β-cortolone are an interesting example of the neighboring group effects studied by Winstein and his colleagues (10). These authors found that *trans*-2-acetoxycyclohexyl-*p*-toluenesulfonate was converted to *cis*-cyclohexane-1,2-diol diacetate by solvolysis with potassium acetate in acetic acid containing a small amount of water. When 3α,17α,21-triacetoxy-20β-*p*-toluenesulfonoxypregnane-11-one (VI) was heated with potassium acetate in aqueous acetic acid, acetyl migration with inversion occurred and the product obtained in high yield was cortolone triacetate

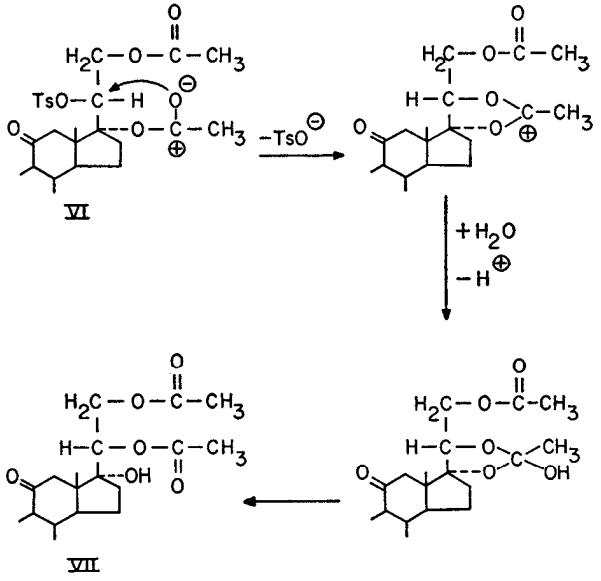

FIG. 3

(VII) (see Fig. 2). The importance of the neighboring group effect is emphasized by the finding that when 3α,21-diacetoxy-17α-hydroxy-20β-*p*-toluenesulfonoxypregnane-11-one was treated with anhydrous potassium acetate in glacial acetic acid the product obtained was β-cortolone triacetate. The intermediate formation of the 17α,20α-epoxide (VIII) in this reaction is strongly suggested by the isolation of this product when 3α,21-diacetoxy-17α-hydroxy-20β-*p*-toluenesulfonoxypregnane-11-one was hydrolyzed with aqueous alkali. Similarly, alkaline hydrolysis of the triacetoxy tosylate (VI), followed by acetylation, yielded the epoxide, VIII. In view of the studies of Winstein and coworkers and the results reported in this investigation, the plausible mechanism illustrated in Fig. 3 can be advanced for the acetyl migration, with inversion of configuration.

In view of the high yield and general ease of operation, the procedure affords a facile synthesis for cortolone, an otherwise relatively inaccessible metabolite. Reduction by means of lithium aluminum hydride formed cortol, quantitatively the most important metabolite of the group studied. These simple and efficient syntheses should make the biochemical investigation of these substances an attractive field for further exploration.

SUMMARY

1. Four new metabolites of adrenal hormones have been isolated from human urine after ACTH administration and identified by isotopic dilution after a tracer dose of hydrocortisone-4-C^{14}. The compounds obtained were (1) pregnane-3α,11β,17α,20α,21-pentol; (2) 3α,17α,20α,21-tetrahydroxypregnane-11-one; (3) pregnane-3α,11β,17α,20β,21-pentol; (4) 3α,17α,20β,21-tetrahydroxypregnane-11-one. Trivial names were suggested for these products.

2. On the basis of the isotopic dilution studies it was estimated that these compounds represent a minimum of 30 per cent of the metabolites of hydrocortisone found in the neutral ether-soluble extract of human urine after hydrolysis of their conjugates by means of beef liver β-glucuronidase.

3. 3α,17α,20α,21-Tetrahydroxypregnane-11-one was isolated from human urine after administration of 100 mg. of cortisone-*t* by vein. It was estimated that the metabolite represented about 6 per cent of radioactivity in the neutral ether-soluble fraction after hydrolysis by means of beef liver β-glucuronidase.

4. A novel and efficient synthesis for 3α,17α,20α,21-tetrahydroxypregnane-11-one was described. The procedure is based upon the solvolysis of 3α,17α,21-triacetoxy-20β-*p*-toluenesulfonoxypregnane-11-one with aqueous acetic acid in the presence of potassium acetate. The reaction is an example of "neighboring group effect" in an acetyl migration with inversion of configuration.

5. The preparation of the related compounds, 3α,21-diacetoxy-17α-hydroxy-20β-*p*-toluenesulfonoxypregnane-11-one, 3α,21-diacetoxy-17α, 20α-epoxypregnane-11-one, and pregnane-3α,11β,17α,20α,21-pentol has been described.

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