hypothesis that *A. xylinum* is a phylogenetic ancestor of *A. suboxydans* thus might imply only that *A. xylinum* has been able to undergo consecutive loss mutations of two non-essential enzyme systems. An opposing view, namely that evolution proceeded from a simple state to a more complex one, may be more attractive on philosophical grounds. In that case, the interesting possibility has to be considered that the ancestor of *Acetobacter* was equipped with a mutative potential so broad as to comprise within its range two enzyme systems (respectively for cellulose synthesis and acetate oxidation) which are facultative in *Acetobacter* but have elsewhere assumed cardinal roles in cellular survival.

SUMMARY

1. A cell-free extract of *Acetobacter xylinum* failed to form cellulose from a range of tested substrates.

2. The extract (with added phenazine methochloride) oxidized glucose aerobically to gluconate. When this system was further supplemented with diphosphopyridine nucleotide, it oxidized gluconate to oxogluconate (largely 2-oxogluconate).

3. The extract with adenosine triphosphate oxidized glucose aerobically to carbon dioxide. The extract contained kinases (glucokinase and gluconokinase), phosphoglucomutase, a complete pentose-cycle set of enzymes, and a set of enzymes by which triose phosphate could be converted into pyruvate.

4. A scheme of alternate pathways for glucose metabolism that can be operated in an *A. xylinum* cell is proposed. The scheme shows a pentose cycle which has several ports of entry and exit. The latter lead from the pentose cycle into a citrate cycle.

5. Biochemical evolution in the genus *Acetobacter* is briefly discussed.

Note added in proof. See Colvin, J. R. (1957). *Arch. Biochem. Biophys.* **70**, 294. Glaser, L. (1957). *Biochim. biophys. Acta*, **75**, 436. Greathouse, G. A. (1957). *J. Amer. chem. Soc.* **79**, 4503.

REFERENCES

De Ley, J. (1954). *Enzymologia*, **17**, 55.
De Moss, R. D. & Gibbs, M. (1955). *J. Bact.* **70**, 730.
Dische, Z. (1955). *Meth. biochem. Anal.* **2**, 251, 327.
Entner, N. & Doudoroff, M. (1952). *J. biol. Chem.* **196**, 853.
Fewster, J. A. (1956). *Biochem. J.* **63**, 26P.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Hauge, J. G., King, T. E. & Cheldelin, V. H. (1955). *J. biol. Chem.* **214**, 11.
Heath, E. C., Hurwitz, J. & Horecker, B. L. (1956). *Abstr. Pap. Amer. Chem. Soc.* **130**, 45c.
Hestrin, S., Aschner, M. & Mager, J. (1947). *Nature, Lond.*, **159**, 64.
Kitos, P. A., King, T. E. & Cheldelin, V. H. (1956). *Fed. Proc.* **15**, 289.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 805.
LePage, G. A. (1949*a*). *Manometric Techniques and Tissue Metabolism*, 2nd ed., p. 187. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publ. Co.
LePage, G. A. (1949*b*). *Manometric Techniques and Tissue Metabolism*, 2nd ed., p. 191. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publ. Co.
Lowry, D. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Roe, J. H., Epstein, J. H. & Goldstein, J. P. (1949). *J. biol. Chem.* **178**, 839.
Roe, J. H. & Papadopoulos, M. M. (1954). *J. biol. Chem.* **210**, 703.
Schramm, M., Gromet, Z. & Hestrin, S. (1957*a*). *Nature, Lond.*, **179**, 28.
Schramm, M., Gromet, Z. & Hestrin, S. (1957*b*). *Biochem. J.* **67**, 669.
Schramm, M. & Hestrin, S. (1954). *J. gen. Microbiol.* **11**, 123.
Schramm, M. & Racker, E. (1957). *Nature, Lond.*, **179**, 1349.
Shimwell, J. L. (1956). *J. Inst. Brew.* **62**, 339.
Shimwell, J. L. (1957*a*). *J. Inst. Brew.* **63**, 44.
Shimwell, J. L. (1957*b*). *J. Inst. Brew.* **63**, 45.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Vandemark, P. & Fukui, G. M. (1956). *J. Bact.* **72**, 610.
Wood, W. A. (1955). *Bact. Rev.* **19**, 222.

The Excretion of *allo*Tetrahydrocortisol in Human Urine

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It is now agreed that cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione; hydrocortisone) is the principal glucocorticoid hormone secreted by the normal human adrenal cortex (Mason & Sprague, 1948; Bush & Sandberg, 1953; Romanoff, Hudson & Pincus, 1953; Sweat, Abbott, Jeffries & Bliss, 1953; Roberts & Szego, 1955). As with other steroids having the Δ^4-3-keto group in ring A, the principal known metabolites of cortisol are steroids in which this group has been reduced to a saturated 3-hydroxyl group (Fig. 1) (Lieberman & Teich, 1953; Fukushima *et al.* 1955). Although four possible stereoisomeric 3-alcohols exist, only one has been found so far among the C_{21} metabolites of cortisol

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(and cortisone), namely that having the configuration 3α-OH-5β(H). On the other hand, all but one of the known saturated 3-alcohols found in adrenal-gland extracts have the configuration 3β-OH-5α(H) (Reichstein & Shoppee, 1943). Klyne (1954) has pointed out that most of the natural steroids of this type fall into these two isomeric classes, which are the two in which the 3-hydroxyl group is in the equatorial, and thermodynamically more stable, configuration. By contrast, C_{21} steroids lacking a 17-hydroxyl group (Dorfman, 1954*a*; Dorfman & Ungar, 1953), and C_{19} steroids, when containing a Δ^4-3-keto group, are reduced to a mixture of isomers in which both 3α-OH-5α(H) and 3α-OH-5β(H) configurations make up appreciable proportions of the mixture (Dorfman, 1954*a*; Lieberman & Teich, 1953; Roberts & Szego, 1955). The relative proportions of such isomers have become of great interest and of some practical importance, since Dorfman (1954*a*, *b*, 1955) showed that they were determined mainly by substituents in other parts of the steroid nucleus. Thus it is possible on Dorfman's theory to calculate the relative secretion rates of the different steroid hormones having a Δ^4-3-keto group in man by estimating the appropriate isomers among their known urinary metabolites. In addition Dorfman (1954*a*) has used some of his findings to suggest that the reactions involved in the metabolism of cortisol to C_{19} metabolites *in vivo* take place in a particular sequence. The theory, however, depends much upon the inability of previous workers to demonstrate any appreciable quantities of a 3α-OH-5α(H) steroid among the major metabolites of cortisone or cortisol (e.g. Burstein, Savard & Dorfman, 1953).

In an examination of the reducing steroids of several hundred urine samples by an extension of methods described earlier (Bush, 1952, 1954; de Courcy, Bush, Gray & Lunnon, 1953) a reducing substance, which had an R_F slightly greater than that of tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one) and which was not present in the urine of patients with adrenal insufficiency (Bush & Lovell, unpublished work; Bush & Willoughby, 1957), was noticed in nearly all of the samples. In this paper evidence will be given that this substance is *allo*tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5α-pregnan-20-one) and that it is a normal metabolite of cortisol and cortisone in man. This substance has long been known as a component of adrenal-gland extracts (Wintersteiner & Pfiffner, 1935; Reichstein, 1936; Mason, 1936, 1938; Kuizenga, 1939) and recently has been shown to be a metabolite of cortisol in isolated perfused rat livers (Caspi, Levy & Hechter, 1953; Caspi & Hechter, 1954). It is peculiar in being one of the few natural C_{21} steroids of animal origin having the axial configuration of its 3-hydroxyl group.

EXPERIMENTAL

Materials

Methanol. This was heated under reflux for 1 hr. with 25 g./l. of KOH and 20 g./l. of granulated zinc. After being filtered, it was distilled slowly through a 25 cm. fractionating column. It was stored in brown foil-stoppered bottles.

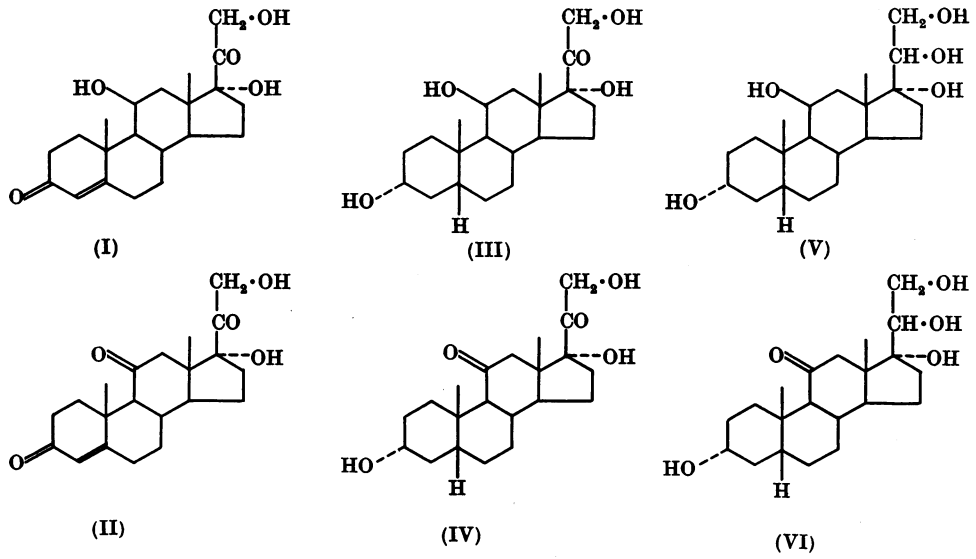

Fig. 1. Cortisol and its main known metabolites in urine. (I) cortisol; (II) cortisone; (III) tetrahydrocortisol; (IV) tetrahydrocortisone; (V) cortol; (VI) cortolone. The last two compounds are excreted each in two forms isomeric at C-20.

Ethanol. This was heated under reflux for 2 hr. with 5 g. of 2:4-dinitrophenylhydrazine and 10 ml. of conc. HCl/l. It was then cooled, filtered and distilled through the 25 cm. column. It was redistilled through the same column at 200 ml./hr.

Benzene. Benzene (750–1500 ml.) was filtered slowly through 100 g. of silica gel (chromatographic grade, Hopkin and Williams Ltd.) holding 20 ml. of conc. H_2SO_4 on a sintered-glass funnel (porosity 2, sintered surface 12 cm. in diameter). After being washed with water and dried over $CaSO_4$ it was distilled slowly through the 25 cm. column and stored in brown foil-stoppered bottles.

Light petroleum (b.p. 100–120°). This was purified in the same way as benzene.

Ethyl acetate. This was washed with 0·25 vol. of 10% Na_2CO_3 and 0·25 vol. of water immediately before use. The wet solvent was used for extracting aqueous phases; for extracting or transferring solid residues the washed solvent was dried over Na_2SO_4 immediately before use.

Ether. This was washed immediately before use with 0·25 vol. of saturated aqueous ferrous sulphate and 0·25 vol. of water. It was dried or used wet as for ethyl acetate.

Pyridine (*A.R.*). This was stored in dark bottles over barium oxide.

m-*Dinitrobenzene.* This was purified by sublimation at atmospheric pressure.

Blue tetrazolium chloride [3:3′-dianisolebis-4:4′-(3:5-diphenyl)tetrazolium chloride]. No satisfactory sample could be obtained from commercial houses, so the reagent was prepared by the method of Rutenburg, Gofstein & Seligman (1950). In the early part of the work an excellent sample, kindly supplied by Dr J. Walker (National Institute for Medical Research), was used.

Sodium bismuthate. Before use each batch was tested by oxidizing 50–100 μg. of cortisol and examining the products chromatographically. Only those batches giving full yields of 11β-hydroxyandrost-4-ene-3:17-dione were used.

Ethanolic potassium hydroxide. A.R. KOH was dissolved in purified ethanol and titrated with HCl. It was then adjusted to 3N and used at once. In the early part of the work pellets were used, but much better results have been obtained recently by using the stick form. Poor results seemed to be due to the frequent presence of brown-stained pellets (iron?) in so-called A.R. grades; sticks with brown stains were less frequent and much easier to detect; batches with stained sticks were rejected.

Blue tetrazolium reagent. Freshly purified blue tetrazolium chloride was made up in purified ethanol as a 1% (w/v) solution and kept in the refrigerator. The stock solution was stable for at least 3 months. The reagent was prepared immediately before use by mixing 2 vol. of stock solution with 5 vol. of water and adding 3 vol. of 4N-NaOH. The reagent was stable enough for use up to 0·5 hr. after making.

Glucuronidase and sulphatase preparations. Roman snails (*Helix pomatia*) were obtained from Watkin and Doncaster (Welling, Kent) and fed on filter paper in a warm room until they were lively. They were then drowned for 15–18 hr. in a closed bucket. The shells were clipped away with strong scissors and an incision 2–3 cm. long made in the back, starting behind the head. The alimentary canal was then allowed to hang from the incision over a small conical flask and snipped open, when the viscous brown succus entericus drained into the flask. The juice was collected from a hundred snails before being mixed and assayed for glucuronidase by the method of Talalay, Fishman & Huggins (1946). It usually contained 60,000–90,000 Fishman units/ml., each snail yielding about 0·7 ml. The mixed enzyme preparation lost only 20% of its activity after being stored in the refrigerator for 7 months. The mixed preparation did not metabolize or destroy added steroids of the type concerned in this investigation and we did not find it necessary to purify our preparation (cf. Henry & Thevenet, 1952). Sulphatases were not assayed, but it was shown in tests on pure dehydro*epi*androsterone (DHA) sulphate that complete hydrolysis of this substance occurred in sodium acetate buffer under our conditions. With urine, hydrolysis of DHA was not complete, but the inhibition was less severe than was expected from the results of Dodgson & Spencer (1953) and was not increased by raising the phosphate concentration. The yield of dehydro*epi*androsterone from its sulphate in urine was 55% by the method described below, and did not differ significantly with different samples of urine.

Filter paper for chromatography. Whatman no. 3 MM and no. 2 were used either as strips or sheets. Before use the strips (22½ in. × 1 in.) of no. 2 and of no. 3 MM (22½ in. × 2 in.) were washed chromatographically (Isherwood & Hanes, 1953) in batches of 100–200 by using successively ethanol–aq. NH_3 soln. (sp.gr. 0·88) (1:1, v/v), ethanol and ether–ethanol (1:1, v/v). After being dried in the fume cupboard they were stored in the dark, wrapped in aluminium foil. When used for isolating material for ultraviolet spectroscopy, the strips were given an additional wash shortly before use by extraction under reflux in a Soxhlet apparatus with benzene–methanol (1:1, v/v).

METHODS

Medical. Patients under treatment with adrenocorticotrophic hormone (ACTH) were given 25 i.u. of ACTHAR gel (Armour) intramuscularly, at 6.0 a.m. and 6.0 p.m. Cortisone was given orally in tablets usually 3–4 times daily; doses ranged from 25 to 200 mg./day. Normal subjects included staff, and patients not seriously ill and not suffering from endocrine disease. Other subjects were patients suffering from various degrees of hirsutism, or mild to moderate Cushing's syndrome.

Urine collection. Samples were collected between 6.0 a.m. and 6.0 a.m. in 2·5 l. bottles containing 100–150 ml. of toluene. The urine was passed into the bottle or transferred to it immediately after each specimen had been passed. The 24 hr. samples were transferred to the cold room (2–4°) within 6 hr. of completion.

Hydrolysis of steroid conjugates. A known fraction (usually 0·05 or 0·2 of a 24 hr. sample) of each urine sample was heated on a boiling-water bath for 20 min. in a conical flask, after it had first been checked that the pH lay between 5·0 and 6·5. The sample was then cooled in tap water and made up to the original volume. 5M-Acetate buffer (0·1 vol.) at pH 4·8 was added and the pH of the sample brought to 4·8 by adding 0·5M-acetic acid and sodium acetate. Succus entericus of *Helix pomatia* (0·02 vol.) was added (Henry & Thevenet, 1952; Jarrige & Henry, 1952). After mixing, the sample was incubated at 45° for 16–18 hr. Tests in which urine samples were incubated, extracted and then buffered and reincubated with fresh enzyme solution showed that

glucuronosides of the principal known steroid metabolites (C_{21} and C_{19}) were completely hydrolysed by this method. Sulphates were only partly hydrolysed (see above) but the yield of DHA from its sulphate was surprisingly constant at 50–60% (i.e. yield over whole procedure including extraction, washing and chromatography). This recovery figure was obtained both by adding pure DHA sulphate to urine samples and by comparing the yield of samples treated enzymically with the yield from portions of the same samples treated by the method of Paterson & Swale (1953).

Extraction. The urine was extracted, after incubation, in cylindrical separating funnels twice with 3 vol. of ether–ethyl acetate (2:1, v/v). The combined extracts were washed three times with 0·05 vol. of N-NaOH and once with 0·05 vol. of water. Two drops of acetic acid were added and the extract was partially dried by filtering slowly through approx. 5 g. of anhydrous Na_2SO_4. It was then distilled to approx. 0·5 ml. on a water bath at 45° by using a fine capillary leak and a high-capacity condenser with a Hivac pump. The extract was then transferred with ethyl acetate and methanol to a 15 ml. conical centrifuge tube. This solution was evaporated to dryness with a fine jet of air (filtered through pellets of sodium hydroxide and glass wool), with the tube immersed in water at 30–40°. No artifacts or serious losses were noticed with this method as long as the air was filtered, the solvents were purified as described and the extracts well washed.

Chromatography. The general procedure was as described by Bush (1952), but most of the work was done at room temperature with the slightly modified systems of Bush (1954). Solvent systems used were: (1) benzene–methanol–water (2:1:1, by vol.); (2) light petroleum–benzene–methanol–water (66:33:80:20, by vol.); (3) light petroleum–methanol–water (25:24:1, by vol.).

Nearly all the material used in this work was isolated from fractions used for the estimation of several classes of steroids. The urine extracts were therefore subjected to a preliminary chromatogram on strips of Whatman no. 3 MM paper 22½ in. × 2 in. by a single-length run in system 2. The strips were then cut into three parts and eluted chromatographically with ethyl acetate–methanol (2:1, v/v). The most polar fraction (fraction 3, Fig. 2) was used for the present study. The details of the chromatography and reactions used to identify *allo*tetrahydrocortisol are described below.

Quantitative estimations. Fraction 3 was evaporated to dryness with a filtered air jet after elution from the preliminary chromatogram on Whatman no. 3 MM paper, and dissolved in 1 ml. of ethanol in a glass-stoppered test tube (3 ml. capacity). A volume equivalent to 0·01 of a 24 hr. urine sample was deposited over the whole width of a 2·5 cm. wide strip of Whatman no. 2 paper in a zone about 4·5 cm. long. It was then concentrated by running up with ethyl acetate–methanol (2:1, v/v), to the starting line (Bush, 1952). After equilibration for 3 hr. in system 1 such strips were run for 15–18 hr. and then dried in the fume cupboard for 0·5 hr. or more. The dry strip was then passed twice over blue tetrazolium reagent in a 10–20 cm. diameter watch glass so that it was wetted from the underside only. Each passage over the surface of the reagent took 20 sec. for a 40 cm. strip of paper and was made in the same direction (i.e. not a pendular movement) at a regular speed. The wet strips were then hung horizontally for 20 min. in still air. This way of dipping and hanging the strips was essential to obtain a constant blank value when the strip was scanned in the absorptiometer. The damp strips were then hung vertically in a tank into which was passed a steady stream of SO_2 from a siphon. The concentration of SO_2 was not critical as long as it was sufficient to cause a spot of B.D.H Universal Indicator at the top of the strip to turn red within 1–2 sec. of putting the strip in the tank. After 20 min. in SO_2 the strips were removed and dried in air for at least 2 hr. The dry strips were then mounted in the enlarged carrier of an EEL Electrophoretic Scanner (Evans Electroselenium Ltd., Essex) specially modified to take 45 cm. strips. The cell was screened with a card diaphragm having a slit 8 mm. horizontally × 22 mm. vertically (i.e. with 1·5 mm. cut off each edge of the strip), and a sheet of Ilford Microfilter no. 4 (Minus Blue). This filter transmits 5300 Å to the infrared. Transmission falls off smoothly with decreasing

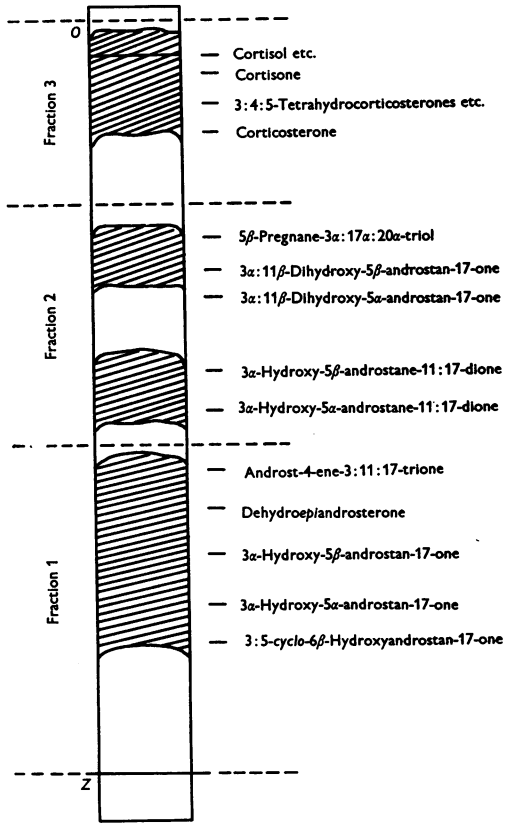

Fig. 2. Preliminary fractionation of urine extracts. Whatman no. 3 MM paper was equilibrated for 3 hr. and run for 1·75 hr. in system 2. The positions of the main urinary neutral steroids are shown on the right, and the fractions taken for subsequent quantitative chromatography on the left. The paper is cut at the dotted lines for eluting the fractions. *O*, origin; *Z*, solvent front; *OZ* measures 40 cm. at 21–24°. The shaded zones are those obtained when a typical urine extract is run and examined with alkaline *m*-dinitrobenzene (fractions 1 and 2) and blue tetrazolium reagent (fraction 3).

wavelength to a value of 0·1% at 5000 Å. Transmission is negligible in the ultraviolet range. The strips were then 'scanned' by hand at intervals of 1–2 mm. The curves obtained were then compared with the strips, the peaks marked off, and a base line was ruled for each peak, by using a fixed convention for all strips. Overlapping peaks were 'split' into their components by careful reconstruction, the profiles from chromatograms of pure steroids and subtraction being used to obtain the profile of the adjacent compound. Chromatograms run for 15–18 hr. were sufficient to give complete separation, by inspection, of the compounds considered here, but slight overlap of the profiles was usually found on scanning. Measurements were not considered reliable if overlap made reconstruction difficult; this, however, was not common unless the room temperature varied greatly during chromatography or some other obvious fault occurred. The areas of each (reconstructed) peak were then measured with a planimeter, and the quantities read off from a calibration curve, constructed similarly from chromatograms of known quantities of pure steroids. A calibration curve was made with four or more quantities of appropriate standards for each batch of estimations. The error of this method has not yet been examined statistically, but during the period of this study, with four-point calibration curves over the range 5·0–80 μg., the great majority of points lay within $\pm 5\%$ of the mean linear regression of quantity on optical density, and no points lay more than $\pm 10\%$ from this line.

A fuller account of this method, its sources of error and its application to other types of steroid is in preparation. Richardson, Touchstone & Dohan (1955) have described a similar method for reducing steroids by means of an adaptor on the Beckman Spectrophotometer.

Methods of detection on chromatograms. Reducing steroids were detected with blue tetrazolium reagent as above, the second dip and treatment with SO_2 being omitted. Δ^4-3-Oxo steroids were detected by the NaOH fluorescence test (Bush, 1952) with 2·7 N-NaOH containing 0·2 ml. of 0·5% (w/v) ethanolic blue tetrazolium chloride/90 ml. of NaOH. 17-Oxo steroids were detected by dipping the chromatograms over the surface of a reagent, made up immediately before use by adding 1 vol. of ethanolic 3 N-KOH to 2 vol. of freshly made 2% (w/v) *m*-dinitrobenzene in ethanol. The wet strip was then dried for 10–20 sec. in the draught of a fume cupboard and finally exposed for 80 sec. to a battery of five electric-fire elements, at a distance such that an ordinary mercury thermometer read 45–47°. Steroids with a glycerol or 17:20-diol side-chain were detected by the method of Bush (1955).

Acetylation. Dry residues containing 20–50 μg. of steroid were dissolved in 2 drops of acetic anhydride and 4 drops of pyridine in a stoppered tube 10 mm. × 12 mm. and left in the dark in a desiccator. The material was then dissolved in ethyl acetate and washed with small volumes of N-Na_2CO_3, N-HCl and water. The washed solution was filtered slowly through 0·5 g. of anhydrous Na_2SO_4 and evaporated with an air jet. The residue was dissolved in ethanol for deposition on chromatograms.

Oxidation with sodium bismuthate (Norymberski, 1952, 1955). The dried material was dissolved in 0·5 ml. of 50% acetic acid and 25 mg. of $NaBiO_3$ added. The suspension was shaken mechanically for 1 hr. in a 100 mm. × 12 mm. stoppered tube screened with black paper. Water (5 ml.) and 10 ml. of saturated $NaHCO_3$ were added and the mixture was extracted twice with 45 ml. of ethyl acetate–ether (1:2, v/v). This extract was filtered slowly through 1 g. of anhydrous Na_2SO_4 and evaporated under reduced pressure on a water bath at 45°.

Oxidation with chromic acid. (Lieberman, Katzenellenbogen, Schneider, Studer & Dobriner, 1953). 17-Oxo steroids and their esters were dissolved in 0·3 ml. of acetic acid and 0·2 ml. of 2% (w/v) aqueous CrO_3 was added. After mixing, the tube (100 mm. × 12 mm.) was stoppered and left at room temperature in the dark for 15–18 hr. It was then diluted with 2·0 ml. of water and extracted with 3 ml. of ethyl acetate. The extract was washed twice with 1 ml. of saturated $NaHCO_3$ and once with 1 ml. of water. After being filtered slowly through anhydrous Na_2SO_4 the extract was evaporated with an air jet or under reduced pressure and dissolved in ethanol for deposition on chromatograms.

21-Acetoxy steroids were dissolved in 0·2 ml. of acetic acid and 0·1 ml. of 1·5% aqueous CrO_3 was added. The stoppered tube was left in the dark for 2·5 hr. and then an ethyl acetate extract prepared as above.

Saponification. 21-Acetoxy steroids were dissolved in a small volume of 0·4% methanolic $KHCO_3$ (w/v), and the solution was saturated with N_2 and left for 8 hr. at room temperature in the dark (Meyer, 1953). The free steroid was extracted with ethyl acetate and the extract washed with water, filtered through anhydrous Na_2SO_4 and evaporated for chromatography, as above. Little hydrolysis of 3-acetoxy groups occurred with this method.

3-Acetoxy groups were hydrolysed by dissolving the steroid in 0·5–2·0 ml. of 0·067 N-NaOH in 80% methanol and leaving in the dark under N_2 for 21 hr. The steroid was then extracted as above.

Absorption spectra. Absorption spectra in concentrated H_2SO_4 were obtained by the method of Zaffaroni (1950, 1953), care being taken to standardize the time at which each wavelength was read. Both known and unknown steroids were treated with H_2SO_4 after elution from a chromatogram, and compared against a blank tube containing the dried eluate from a similar area of the chromatogram containing no added steroid. Despite these precautions, material giving a strong peak at 320 mμ was troublesome in the eluates from chromatograms run in system 2. Good results were obtained with material from chromatograms run in system 1.

Ordinary ultraviolet spectra were measured in ethanol. All spectra were measured on the Unicam spectrophotometer.

Identification of allo*tetrahydrocortisol*

The schemes of reactions and chromatograms used for this identification are given in Figs. 3 and 4.

Pool 1. Residues of fraction 3 from six 24 hr. urine samples from a patient with mild adrenogenital syndrome were pooled and run on Whatman no. 3 MM paper for 5 hr. in system 1. The unknown reducing substance was eluted and run on Whatman no. 2 paper for 15 hr. in system 1. After elution of the appropriate zone, a fraction of the eluate calculated to contain 25 μg. was oxidized with $NaBiO_3$ and the product run for 16 hr. in system 2. Half the chromatogram was treated with alkaline *m*-dinitrobenzene, which gave a purple spot identical in position and colour with that given by 3α:11β-dihydroxy-5α-androstan-17-one on the same sheet. The appropriate area of the other half of the sheet was eluted and the material acetylated in parallel

with 3α:11β-dihydroxy-5α-androstan-17-one. The esters were run for 14 hr. with system 3. On being treated with alkaline *m*-dinitrobenzene the two esters gave purple spots 32·6 and 32·8 cm. from the origin, as expected for the 3-acetate of the reference steroid.

The remainder of the original substance was acetylated and run in system 2 (single-length run). A reducing substance was detected with blue tetrazolium running just behind the diacetate of tetrahydrocortisol. (Traces of 3α-hydroxy-5β-androstane-11:17-dione were found with the main $NaBiO_3$ oxidation product; these were explained by the presence of traces of tetrahydrocortisone diacetate on the chromatogram of the esters in system 2.)

Pool 2. The unknown substance was obtained as in pool 1 from another patient with mild adrenogenital syndrome and purified by running on Whatman no. 3 MM paper in system 1 for 15 hr. It was then run for 18 hr. on Whatman no. 2 paper in system 1 and treated with blue tetrazolium reagent and with the NaOH fluorescence test. No substance giving any fluorescence with NaOH was found on the chromatogram. On running in system 1 for 36 hr. the substance still gave a single spot with blue tetrazolium reagent.

Pool 3. The unknown substance was prepared as above from the urine of another patient with adrenogenital syndrome. It was then run in parallel with *allo*tetrahydrocortisol for 15 hr. in system 1. After acetylation the substance was run in parallel with *allo*tetrahydrocortisol diacetate in system 2 (single-length run). Both the free compound and its ester moved to positions identical with those of the reference steroids.

A part of the unknown ester was oxidized with chromic acid and run in system 2 (single length) in parallel with *allo*tetrahydrocortisone diacetate (3α:21-diacetoxy-17α-hydroxy-5α-pregnane-11:20-dione). The R_F values of the two substances were identical.

The chromic acid oxidation product of the ester of the unknown substance was then submitted to hydrolysis with methanolic $KHCO_3$ simultaneously with *allo*tetrahydrocortisone diacetate. The products were run in system 2 (single-length run). Both substances gave rise to a reducing substance in the position expected for the 3-monoacetate of *allo*tetrahydrocortisone.

Pool 4. Residues of fraction 3 from five patients with Cushing's syndrome were pooled. The pooled extract was compared with *allo*tetrahydrocortisol by running in system 1 for 36 hr. The urinary reducing substance appeared to be homogeneous and had moved to the same position as the reference steroid.

Pool 5. The unknown substance was isolated from the urine of a patient with adrenogenital syndrome by running fraction 3 on Whatman no. 3 MM paper for 15 hr. with system 1, and eluting. The material was oxidized with sodium bismuthate and part of the oxidation product acetylated in parallel with 3α:11β-dihydroxy-5α-androstan-17-one. The esters were then oxidized with chromic acid and the products were saponified with methanolic NaOH. On running in system 2 (single-length run) both products gave a single spot with alkaline *m*-dinitrobenzene in the position known for 3α-hydroxy-5α-androstane-11:17-dione, and giving the characteristic pinkish colour which partly

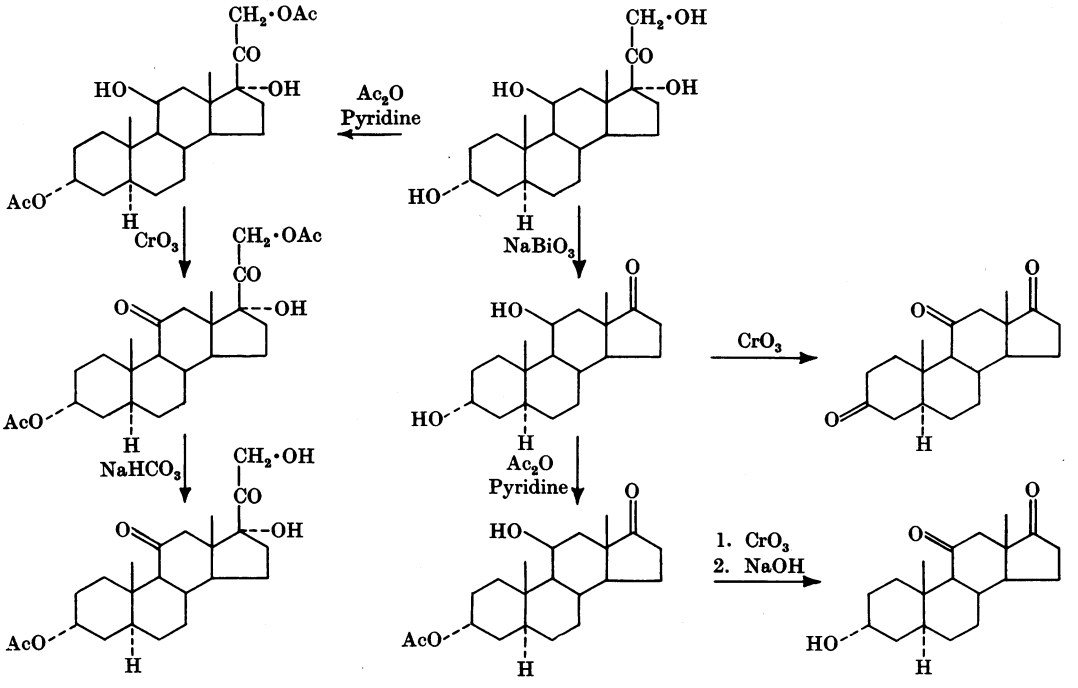

Fig. 3. Scheme of reactions used to identify *allo*tetrahydrocortisol in extracts of urine. In each case the appropriate reference compound was treated with reagents and run on a chromatogram in parallel with the material from urine. In this way relative yields of major and minor products can be compared directly with confidence.

develops in the cold. Traces of androstane-3:11:17-trione and presumed 3α-acetoxy-5α-androstane-11:17-dione were also found with both products.

The remainder of the $NaBiO_3$ oxidation product of the original substance was oxidized with chromic acid for 16 hr. simultaneously with samples of 3α:11β-dihydroxy-5α-androstan-17-one and 3α-hydroxy-5α-androstane-11:17-dione. The three products were then run in system 3 for 15 hr. and the chromatogram was treated with alkaline *m*-dinitrobenzene. All three products gave immediate bluish-purple colours in the cold which intensified on heating, and were at identical distances from the origin. In subsequent tests (Fig. 4) it was shown that the triones isomeric at C-5 are not separated in this solvent system (i.e. 5α-androstane-3:11:17-trione and 5β-androstane-3:11:17-trione).

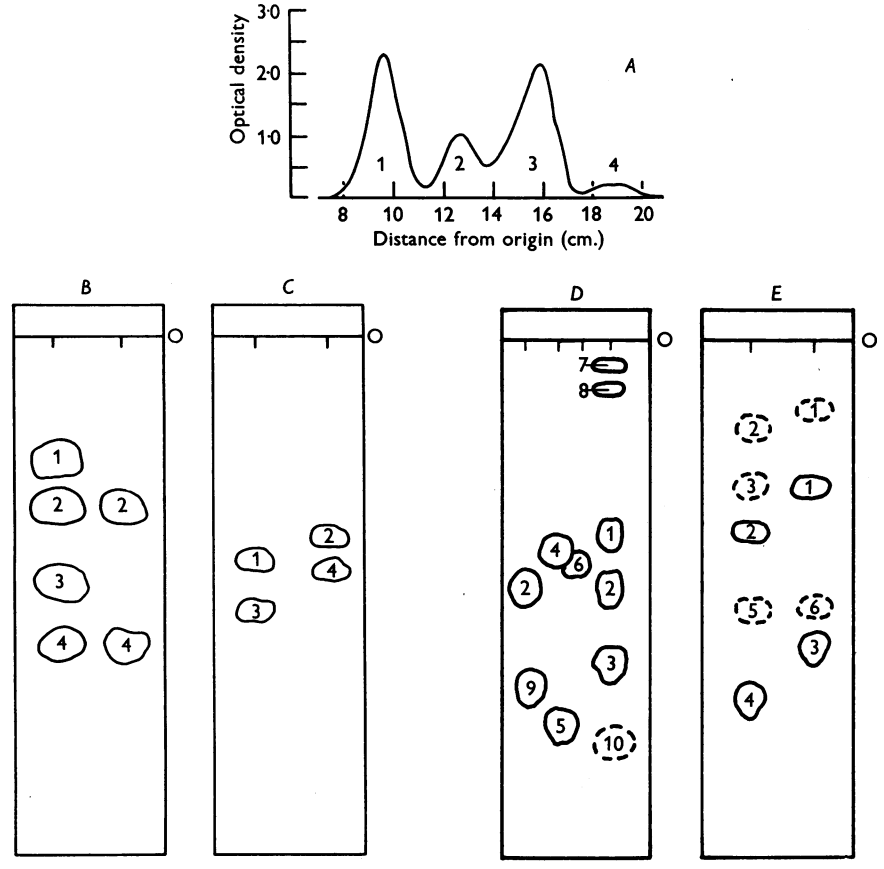

Fig. 4. *A*, Typical scanning record of reducing steroids on a chromatogram of a urinary extract. The strip was equilibrated for 3 hr. and run for 18 hr. in system 1 at room temperature, with Whatman no. 2 paper. 1, tetrahydrocortisol; 2, *allo*tetrahydrocortisol; 3, tetrahydrocortisone; 4, cortisol. Chromatograms *B–E* show typical separations at room temperature. To achieve complete resolution of 5α and 5β epimers, over-run chromatograms (14–18 hr.) were always used. The epimeric (5α- and 5β-)androstanetriones cannot be separated in these systems.

B, 1, tetrahydrocortisol; 2, *allo*tetrahydrocortisol; 3, tetrahydrocortisone; 4, *allo*tetrahydrocortisone (system 1, Whatman no. 2, equilibrated 3 hr., run 18 hr.). O, origin with two tracks of compounds. 2 and 4 run in both tracks.

C, 1, tetrahydrocortisol diacetate. 2, *allo*tetrahydrocortisol diacetate. 3, tetrahydrocortisone diacetate. 4, *allo*-tetrahydrocortisone diacetate (system 2, Whatman no. 2, equilibrated 3 hr., run 4 hr.).

D, 1, dehydro*epi*androsterone. 2, 3α-hydroxy-5β-androstan-17-one. 3, 3α-hydroxy-5α-androstan-17-one. 4, 3β-hydroxy-5α-androstan-17-one. 5, 3β-hydroxy-5β-androstan-17-one. 6, 3α-hydroxy-5β-androst-9(11)-en-17-one. 7, 3α:11β-dihydroxy-5β-androstan-17-one. 8, 3α-hydroxy-5β-androstane-11:17-dione. 9, 6β-hydroxy-3:5-*cyclo*androstan-17-one. 10, 3α-acetoxy-11β-hydroxy-5α-androstan-17-one. Run in system 3 on Whatman no. 2 paper, equilibrated 3 hr.; —, run 16 hr.; - - -, run 14 hr.; O, origin with 4 tracks of compounds.

E, 1, 3α:11β-dihydroxy-5β-androstane-17-one. 2, 3α:11β-dihydroxy-5α-androstan-17-one. 3, 3α-hydroxy-5β-androstane-11:17-dione. 4, 3α-hydroxy-5α-androstane-11:17-dione. 5, 5α-androstane-3:11:17-trione. 6, 5β-androstane-3:11:17-trione. O, origin with two tracks of compounds. Conditions were as for *D*; but —, run 16 hr.; - - -, run 4 hr.

Absorption spectra. Samples of the unknown substance from pools 1–3 were run on specially washed paper (see Materials) and eluted. The diacetate and the chromic acid oxidation product of the diacetate were treated similarly, in parallel with the appropriate reference steroids. Details of the spectra in H_2SO_4 are given in Table 1. Not unexpectedly the agreement between the spectra of the diacetates and their reference steroids was unsatisfactory because of the impurities, described above, in eluates from chromatograms run in system 2, and also because the resolution obtained with the single-length run is not great. The agreement between the free compounds, in which a long run in system 1 could be made, was, however, good. As expected from the absence of fluorescence with NaOH, the original substance in ethanol gave no absorption peak in the ultraviolet.

Table 1. *Absorption spectra in concentrated sulphuric acid*

The diacetates were isolated from chromatograms run with system 2, which gave unexpectedly high blank values with sulphuric acid in the range 240–360 mμ; the differences between reference steroid and isolated urinary material were therefore not considered significant.

Compound	$\lambda_{max.}$ (mμ)
Tetrahydrocortisol	245, 330, 410, 505
*allo*Tetrahydrocortisol (I)	240, 330, 410, 505
Urinary material	240, 330, 410, 505
Diacetate of (I)	240, 285, 342, 415
Diacetate of urinary material	235, 320, 410
*allo*Tetrahydrocortisone diacetate	275, 320, 410
CrO_3 oxidation product of the diacetate of urinary material	240, 317, 410

RESULTS

Excretion rate of allo*tetrahydrocortisol*

Table 2 summarizes the excretion rates of the three reducing steroids believed to be derived from cortisol in man. It is seen that *allo*tetrahydrocortisol is excreted in quantities that are far from insignificant, although less than those of the other two steroids. It is also clear that the ratio between the (5β)-pregnane metabolites and *allo*tetrahydrocortisol [(5α)-pregnane] is not greatly different in the different groups of subjects studied here.

In Fig. 5 the results are plotted as a regression of the excretion rates of the (5α)-pregnane metabolite on the sums of the excretion rates of the (5β)-pregnane metabolites in each of 52 urine samples. If y is the excretion rate of *allo*tetrahydrocortisol and x the sum of the excretion rates of tetrahydrocortisol and tetrahydrocortisone, the regression of y on x is given by the equation $y = 0{\cdot}199x - 0{\cdot}1625$. The regression coefficient is 0·924 ($P \leqslant 0{\cdot}001$); the deviation of the point of intersection of the x and y axes ($a = -0{\cdot}1625$) is not significant ($P \geqslant 0{\cdot}5$).

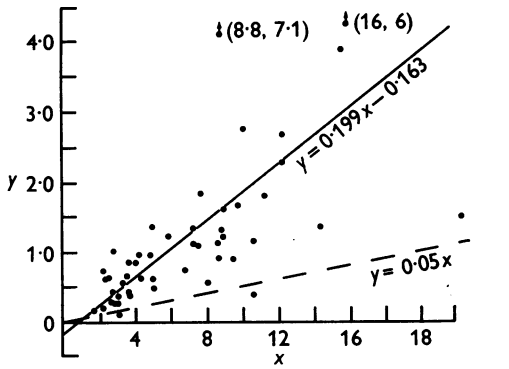

Fig. 5. Regression of excretion rates of *allo*tetrahydrocortisol (y) on the summed excretion rates of tetrahydrocortisol and tetrahydrocortisone (x), in mg./24 hr. Solid line is for $y = 0{\cdot}199x - 0{\cdot}163$. Interrupted line is for $y = 0{\cdot}05x$, as expected from Dorfman's (1954*a*) theory.

Table 2. *Excretion rates of the main* 3:4:5*-tetrahydro metabolites of cortisol*

Rates (mg./24 hr.) are given as means with ranges in parentheses and are corrected for the analytical losses usual in the method of estimation. The mean ratios 5α(H) metabolite/5β(H) metabolites are given in the last column.

Subjects	No.	Tetrahydrocortisol mean (range)	*allo*Tetrahydrocortisol mean (range)	Tetrahydrocortisone mean (range)	5α(H)/5β(H)
Normal men and women	14	2·0 (1·1–4·8)	0·8 (0–2·8)	3·4 (1·0–10·4)	0·165
Idiopathic hirsutism (women)	7	1·8 (0·9–4·5)	0·7 (0·3–1·6)	2·3 (1·1–4·6)	0·202
Cushing's syndrome (mild)	4	4·15 (3·5–5·3)	1·2 (0·9–1·7)	4·85 (3·9–5·7)	0·42
Adrenogenital syndrome	11	2·0 (0·4–3·3)	0·75 (0·2–1·8)	2·7 (1·1–4·5)	0·173
Obesity and hypertension	1	1·7	0·6	1·9	0·166
Various women with hirsutism or adrenogenital syndrome on oral cortisone	10	3·55 (1·8–4·0)	1·2 (0·6–2·3)	6·0 (1·8–10·5)	0·162
Women with hirsutism or adrenogenital syndrome on ACTH gel, intramuscular	5	10·6 (4·7–18·0)	5·7 (1·3–12·4)	9·9 (4·3–17·7)	0·25

While the regression equation and coefficient indicate a highly significant correlation of the excretion of *allo*tetrahydrocortisol with that of tetrahydrocortisol and tetrahydrocortisone, it should be noticed that this is heavily weighted towards significance by a few figures from subjects excreting large quantities of these steroids. Furthermore, separation of the subjects into groups according to their clinical state and treatment reveals significant differences ($P \leqslant 0{\cdot}025$) between the ratio 5α-(H) compound:5β-(H) compounds of the different groups, if these are tested by a test for ordered alternatives rather than against a null hypothesis (Jonckheere, 1954). Thus this ratio is lower in a small group of cases of Cushing's syndrome, and higher among the hirsute females, than in the normal group (mean ratios 0·142, 0·202, 0·165). A striking finding was that treatment of the hirsute group with cortisone lowered this ratio to normal (mean 0·162), whereas treatment with ACTH increased it still further (mean 0·250), both differences significant at $P \leqslant 0{\cdot}02$. There is no question of a systematic error underlying this finding, since the total excretion rates were large in both groups, the mean total excretion rates (reducing steroids) of both groups being larger than in the untreated patients. There are two possible explanations of this finding, but further work is needed to decide between them with confidence.

The first is that the phenomenon is due to a difference in the metabolism of cortisol (or cortisone) by the three groups of subjects, which is shown up by oral administration of cortisone. The major part of the steroid is absorbed via the portal system when administered orally, and if the enzyme system producing the 5α-(H) metabolite was of relatively limited activity, in contrast to that producing the 5β-(H) metabolite, the proportion of 5α-(H) metabolite would fall during oral administration of its precursor. An alternative hypothesis of this type, which would be in accord with the very high substrate specificity of Δ^4-3-oxo steroid reductases (Tomkins, 1957), would be that cortisol, but not cortisone, was reduced to the 5α-(H) metabolite. This type of hypothesis would not, without elaboration, explain the increased proportion of *allo*tetrahydrocortisol excreted during treatment with ACTH. A second type of explanation, accounting for this and the other observations, would be that *allo*tetrahydrocortisol was secreted by the adrenal cortex, in addition to being a metabolite of cortisol, and that the proportion secreted was larger than normal in the hirsute women and lower than normal in the cases of Cushing's syndrome. This hypothesis seems to us rather doubtful but receives some support from the fact that *allo*tetrahydrocortisol and its 11-oxo derivative are found in extracts of adrenal glands, and are the only 3α-OH-5α(H) pregnane derivatives in such extracts (Reichstein, 1936).

None of these three reducing steroids were found in the urine of two patients with Addison's disease, or in the urine of a man who had been bilaterally adrenalectomized, when they had been treated with deoxycorticosterone and salt. In all three cases all three steroids appeared in the urine when cortisone (25–50 mg./day) was given.

DISCUSSION

Identification of allo*tetrahydrocortisol*

The scheme employed has been summarized in Fig. 3, and the chromatograms of the substances and their derivatives involved in this study are shown in Fig. 4. Following the general lines suggested before (Bush & Sandberg, 1953; Bush, 1954) seven derivatives were prepared and compared chromatographically with reference steroids. Not only were the mobilities of the substance and all seven derivatives identical in the three solvent systems employed, but several characteristic details of these mobilities and the reactions of the derivatives are worth emphasizing. Thus proportions of minor products of oxidation, esterification and saponification were exactly the same for reference steroid and urinary material in each case. Highly characteristic also was the fact that, although the free 3α-OH-5β(H) steroids were slightly less polar than the related 3α-OH-5β(H) isomers, the opposite was true for their diacetates. Other features were the reactions given by the 17-oxo steroid derivatives with alkaline *m*-dinitrobenzene. 3α:11β-Dihydroxy-5α-androstan-17-one gave a rather weak purple colour only after heating; 3α-hydroxy-5α-androstane-11:17-dione [like its (5β)-*cis–trans* isomer] gave a strong pinkish colour developing partly in the cold; (5α)-androstane-3:11:17-trione gave a strong bluish colour developing partly in the cold. In all these details the derivatives of the urinary substance agreed exactly with the known reference steroids.

The series of reactions used here subjected each substituent in the steroid nucleus in turn to one or more unequivocal reactions. In this way changes in colour reaction and chromatographic mobility could be interpreted with considerable confidence. The sequence of reactions given in Fig. 3 is based on the reasonable assumption that the material is a steroid, and the reasonable induction from previous work that within limits the identification of steroids by chromatographic mobility and colour reactions is sound (Zaffaroni, 1953; Savard, 1954; Bush, 1954). It is, however, possible to consider the evidence largely without relying upon the assumption that the material is a steroid (see Table 3).

Previous workers have failed to detect *allo*tetrahydrocortisol in human urine, although it has been seen that its excretion rate, though small, is not insignificant compared with tetrahydrocortisol and tetrahydrocortisone, which are well known as urinary metabolites (Schneider, 1950, 1952; Baggett, Kinsella & Doisy, 1953; de Courcy *et al.* 1953; Lieberman *et al.* 1953; Romanoff, Wolf, Constandse & Pincus, 1953; Romanoff & Wolf, 1954; Cope & Hurlock, 1954; Richardson *et al.* 1955). [After the present paper had been submitted for publication Romanoff, Seelye, Rodriguez & Pincus (1957) announced the identification of *allo*tetrahydrocortisol in the urine of normal humans and

Table 3. *Summary of steps in the identification of urinary* allo*tetrahydrocortisol*

In Fig. 3 the series of reactions used is given in terms of the actual steroid derivatives expected from such reactions, and identification of each derivative is based on identical chromatographic mobilities and colour reactions. In this table each functional group is considered separately and without reference to the identity of the derivatives themselves.

Side chain

(1) Oxidized by $NaBiO_3$ to a carbonyl compound.
(2) Oxidized by alkaline blue tetrazolium rapidly in the cold.
(3) Not oxidized to a carbonyl compound by periodate.
(4) Not oxidized by chromic acid after acetylation.
(5) Oxidized by alkaline blue tetrazolium rapidly in the cold after acetylation.
(6) No Zimmermann reaction.
(7) $NaBiO_3$ oxidation product gives purple colour with Zimmermann reagent characteristic of 17-oxo steroids.

The reducing side-chain is linked to the nucleus with a tertiary hydroxyl group (1, 7) but does not contain a hydroxyl group adjacent to this (3). The reducing part contains an easily esterifiable group (4), the ester being labile to cold alkali (5). The only known group meeting all requirements is a dihydroxyacetone side-chain.

11β-Hydroxyl group

(8) Oxidation with CrO_3, after acetylation, causes an increase in R_F of the expected amount with the original substance, and with the derivative given by $NaBiO_3$ oxidation.
(9) The oxidizable group is not protected by acetylation in the cold.
(10) The $NaBiO_3$ oxidation product gives a weak purple colour with the Zimmermann reagent. Oxidation of the group in question gives a derivative giving a strong pink colour in the cold with the Zimmermann reagent.

A strongly hindered secondary hydroxyl group is responsible for these properties (8, 9). All these (8, 9, 10) are highly characteristic of the 11β-hydroxyl group in the natural steroids. No other hydroxyl group in the steroid nucleus has all these properties.

3-Hydroxyl group

(11) An easily esterifiable group exists on the nucleus, since acetylation of the substance causes an increase in R_F greater than that accounted for by the group in the side chain.
(12) The increase in R_F of the substance, and of its $NaBiO_3$ oxidation product, after acetylation, are too small to be due to more than one such group.
(13) The esterified group is hydrolysed by NaOH in the cold but not by $KHCO_3$.
(14) The group is oxidized by CrO_3 to a less polar group which causes a considerable blueing of the colour given by the Zimmermann reagent.

One acetylatable secondary hydroxyl group exists on the nucleus (11, 12, 14). In the steroids only position 3 will satisfy (14). Position 3 satisfies (13).

3α-OH-(5α)H configuration

(15) Oxidation of the second nuclear secondary hydroxyl group causes an increase in R_F which is too small for 3β-OH-5α(H) or 3α-OH-5β(H) and too large for 3β-OH-5β(H) configurations. This increase in R_F is exactly that found with the 3α-OH-5α(H) configuration.
(16) The increase in R_F on acetylation is too small for the 3α-OH-5β(H) configuration.

Only the 3α-OH-5α(H) configuration satisfies all requirements (14, 15, 16).

Absence of other substituents

(17) The substance and all its recognizable derivatives are not extracted by alkali (Na_2CO_3) or acid (HCl) from ethyl acetate.
(18) No derivative given by CrO_3 oxidation has a lower R_F than the starting material of such an oxidation.
(19) The substance has no absorption maximum in visual or ultraviolet range.
(20) R_F values of the substance and all derivatives satisfy the existence of a steroid nucleus and the polar groups shown by the reactions above.

Carboxyl (17), basic (17), phenolic (19), other tertiary hydroxyl groups (18), and conjugated systems (19) are excluded. Exceptionally stable ester groups or very weakly basic groups are not excluded by the reactions above; it is highly unlikely that such groups would not be revealed by differences in R_F or colour reactions of one or more of the seven derivatives prepared.

schizophrenics. Their findings are very similar to those reported here.] Although this could be due to a variety of differences in technique, it seems most likely that incomplete separation from tetrahydrocortisol on chromatograms, coupled with the identical spectra in sulphuric acid of these two *cis–trans* isomers, was responsible. Richardson *et al.* (1955) have pointed out already that the latter factor might have vitiated their identification of tetrahydrocortisol; inspection of their scanning curves suggests that the two epimers would not have been separated. Similarly, the single-length chromatograms of de Courcy *et al.* (1953) were not sufficient to separate either the free compounds or their acetates when mixed in the proportions usual in urine extracts. However, the presence of the (5α)-epimer would have been demonstrated, even if it had not been separated from tetrahydrocortisol, if oxidation with sodium bismuthate had been carried out, since the analogous *cis–trans* epimers among the C_{19} steroids are more easily separated.

Metabolic origin of allo*tetrahydrocortisol*

The direct evidence from patients with adrenal insufficiency, and the significant correlation of the excretion rate of this substance with those of tetrahydrocortisol and tetrahydrocortisone (which are known metabolites of cortisone and cortisol), under a wide variety of conditions, suggest strongly that *allo*tetrahydrocortisol is itself a metabolite of cortisol. The mean ratio of *allo*tetrahydrocortisol (tetrahydrocortisol + tetrahydrocortisone) in the fifty-two urine samples was 0·16 (S.D. 0·08). While this is about three times that expected from the theory of Dorfman (1954*a*), it does not contradict the general rules he has described. However, the wide scatter of the values of this ratio, when measured in single 24 hr. specimens of urine, is similar to that found with other classes of steroid with the same *cis–trans* isomerism (Gallagher, 1954*a*, *b*; Fukushima, Dobriner & Gallagher, 1954; Dorfman, 1954*b*, 1955).

It will be seen from Fig. 4 that *allo*tetrahydrocortisone would not be separated from cortisol on our routine quantitative chromatograms. However, we have evidence that the former substance was not present in more than traces in the urine samples studied here. Measurement of the excretion rate of cortisol was made on many of the specimens of this series, by separate chromatograms and the NaOH fluorescence test (Bush, 1953), by means of visual matching with standards (error ± 20 %). In every case where reducing material was found in this zone, on chromatograms examined by the quantitative blue tetrazolium method, it could all be accounted for by the amount of cortisol determined by the fluorescence method. In most cases the amount of reducing material in this zone was just at or below the limit of measurement with blue tetrazolium (i.e. with 0·01 of 24 hr. sample), so that even in samples where cortisol was not measured separately the amount of *allo*tetrahydrocortisone must have been extremely small. It seems unlikely that the ratio of the 11-oxo to the 11-hydroxyl compound can have exceeded 0·05 in any of our subjects.

This negligible quantity of the 11-oxo compound is remarkable (Bush, 1956) in that the same phenomenon occurs with two other types of 5α(H)-3α:11β-dihydroxy steroids, in contrast with their 5β(H) *cis–trans* isomers. Thus 3α:11β-dihydroxy-5α-androstan-17-one and 3α:11β:21-trihydroxy-5α-pregnan-20-one are found in human urine, but their 11-oxo forms are not present in easily demonstrable quantity (Gallagher, 1954*b*; Dorfman, 1954*b*; Kappas & Gallagher, 1955; Kappas, Dobriner & Gallagher, 1955; Engel, Carter & Springer, 1954; Richardson *et al.* 1955). On the other hand, with the related (5β)-H *cis* isomers the 11-oxo form predominates in a ratio of 2–4. If the enzyme reducing 11-oxo steroids to 11β-hydroxy steroids found in rat-liver homogenates by Hubener, Fukushima & Gallagher (1956), is present in man, then these findings would be explained if the enzyme were capable of acting on 3α-OH-5α(H) steroids as well as on Δ^4-3-oxo steroids. This has not yet been tested, but Hubener *et al.* (1956) have shown that this enzyme cannot attack 3α-OH-5β(H) steroids, which would afford a possible explanation of the predominance of the 11-oxo forms of the 3α-OH-5β(H) steroid metabolites. [Since this paper was submitted, it has been shown that 3α-hydroxy-5α-androstane-11:17-dione is partially reduced to the 11-alcohol in man (Bush & Mahesh, 1957).] The only other explanation for this phenomenon would be that the enzyme responsible for reduction of Δ^4-3-oxo steroids to 3α-OH-5α(H) steroids did not act *in vivo* on 11-ketones, although it acted on 11-deoxy steroids and on 11β-alcohols; while possible, this seems very much less likely than the first hypothesis.

SUMMARY

1. A reducing substance was noticed on chromatograms of urine extracts which had an R_F just greater than that of 3α:11β:17α:21-tetrahydroxy-5β-pregnan-20-one. It was almost invariably present in urine from normal subjects, and from patients with various forms of hyperadrenalism, but absent from patients with Addison's disease or those who had undergone adrenalectomy, unless the latter patients were treated with cortisone.

2. Derivatives were prepared of the reducing substance after isolation from chromatograms, in parallel with known steroids, and identified by

means of chromatographic mobility, colour reactions, and spectra in sulphuric acid. In all respects the substance was found to be identical with 3α:11β:17α:21-tetrahydroxy-5α-pregnan-20-one.

3. Evidence was obtained that the reducing substance is a normal metabolite of hydrocortisone and cortisone in man, previously unrecognized because of its similarity to its 5β-epimer in many respects. This modifies some of the earlier conclusions of Dorfman (1954*a*).

4. Further work is needed to elucidate some unusual features of the excretion rate of the reducing substance in women suffering from various forms of adrenal virilism and hirsutism.

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REFERENCES

Baggett, B., Kinsella, R. A. & Doisy, E. A. (1953). *J. biol. Chem.* **203**, 1013.
Burstein, S., Savard, K. & Dorfman, F. I. (1953). *Endocrinology*, **52**, 448.
Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Bush, I. E. (1953). *J. Endocrin.* **9**, 95.
Bush, I. E. (1954). *Recent Progr. Hormone Res.* **9**, 321.
Bush, I. E. (1955). *Biochem. J.* **50**, xiv.
Bush, I. E. (1956). *Experientia*, **12**, 325.
Bush, I. E. & Mahesh, V. B. (1957). *Biochem. J.* **66**, 56P.
Bush, I. E. & Sandberg, A. A. (1953). *J. biol. Chem.* **205**, 783.
Bush, I. E. & Willoughby, M. (1957). *Biochem. J.* **66**, 28P.
Caspi, E. Y. & Hechter, O. M. (1954). *Arch. Biochem. Biophys.* **53**, 478.
Caspi, E. Y., Levy, H. & Hechter, O. M. (1953). *Arch. Biochem. Biophys.* **45**, 169.
Cope, C. L. & Hurlock, B. (1954). *Clin. Sci.* **13**, 69.
de Courcy, C., Bush, I. E., Gray, C. H. & Lunnon, B. (1953). *J. Endocrin.* **9**, 401.
Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 315.
Dorfman, R. I. (1954*a*). *Recent Progr. Hormone Res.* **9**, 5.
Dorfman, R. I. (1954*b*). *Adrenal Cortex.* 5th Conf. Josiah Macy Jr. Found.
Dorfman, R. I. (1955). *Ann. N.Y. Acad. Sci.* **61**, 291.
Dorfman, R. I. & Ungar, F. (1953). *Metabolism of the Steroid Hormones.* Minneapolis: Burgess Publishing Co.
Engel, L. L., Carter, P. & Springer, M. J. (1954). *Fed. Proc.* **13**, 204.
Fukushima, D. K., Dobriner, K. & Gallagher, T. F. (1954). *J. biol. Chem.* **206**, 845.
Fukushima, D. K., Leeds, N. S., Bradlow, H. L., Kritchevsky, T. H., Stokem, M. B. & Gallagher, T. F. (1955). *J. biol. Chem.* **212**, 449.
Gallagher, T. F. (1954*a*). *Recent Progr. Hormone Res.* **9**, 26.
Gallagher, T. F. (1954*b*). *Adrenal Cortex.* 5th Conf. Josiah Macy Jr. Found.
Henry, R. & Thevenet, M. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 886.
Hubener, H. J., Fukushima, D. K. & Gallagher, T. F. (1956). *J. biol. Chem.* **220**, 49.
Isherwood, F. A. & Hanes, C. S. (1953). *Biochem. J.* **55**, 824.
Jarrige, P. & Henry, R. (1952). *Bull. Soc. Chim. biol., Paris*, **34**, 872.
Jonckheere, A. R. (1954). *Biometrika*, **41**, 133.
Kappas, A., Dobriner, K. & Gallagher, T. F. (1955). *J. clin. Invest.* **34**, 1559.
Kappas, A. & Gallagher, T. F. (1955). *J. clin. Invest.* **34**, 1566.
Klyne, W. (1954). In *Progress in Stereochemistry*, vol. 1, p. 36. Ed. by Klyne, W. London: Butterworths Scientific Publications.
Kuizenga, M. H. (1939). *Endocrinology*, **24**, 526.
Lieberman, S., Katzenellenbogen, E. R., Schneider, R., Studer, P. E. & Dobriner, K. (1953). *J. biol. Chem.* **205**, 87.
Lieberman, S. & Teich, S. (1953). *Pharmacol. Rev.* **5**, 285.
Mason, H. L. (1936). *J. biol. Chem.* **114**, 616.
Mason, H. L. (1938). *J. biol. Chem.* **124**, 459.
Mason, H. L. & Sprague, R. G. (1948). *J. biol. Chem.* **175**, 451.
Meyer, A. (1953). *J. biol. Chem.* **203**, 469.
Norymberski, J. K. (1952). *Nature, Lond.*, **170**, 1074.
Norymberski, J. K. (1955). *Biochem. J.* **60**, 457.
Paterson, J. & Swale, J. (1953). *Mem. Soc. Endocrin.* no. 2, 41.
Reichstein, T. (1936). *Helv. chim. acta*, **19**, 29.
Reichstein, T. & Shoppee, C. W. (1943). *Vitam. & Horm.* **1**, 345.
Richardson, E. M., Touchstone, J. C. & Dohan, F. C. (1955). *J. clin. Invest.* **34**, 285.
Roberts, S. & Szego, C. M. (1955). *Annu. Rev. Biochem.* **24**, 543.
Romanoff, E. B., Hudson, P. & Pincus, G. (1953). *J. clin. Endocrin. Metab.* **13**, 1546.
Romanoff, L. P., Seelye, J., Rodriguez, R. & Pincus, G. (1957). *J. clin. Endocrin. Metab.* **17**, 434.
Romanoff, L. P. & Wolf, R. S. (1954). *Recent Progr. Hormone Res.* **9**, 337.
Romanoff, L. P., Wolf, R. S., Constandse, M. & Pincus, G. (1953). *J. clin. Endocrin. Metab.* **13**, 928.
Rutenberg, A. M., Gofstein, R. & Seligman, A. M. (1950). *Cancer Res.* **10**, 113.
Savard, K. (1954). *Recent Progr. Hormone Res.* **9**, 185.
Schneider, J. J. (1950). *J. biol. Chem.* **183**, 365.
Schneider, J. J. (1952). *J. biol. Chem.* **194**, 337.
Sweat, M. L., Abbott, W. E., Jeffries, W. M. & Bliss, E. L. (1953). *Fed. Proc.* **12**, 141.
Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
Tomkins, G. H. (1957). *J. biol. Chem.* **225**, 13.
Wintersteiner, O. & Pfiffner, J. J. (1935). *J. biol. Chem.* **111**, 599.
Zaffaroni, A. (1950). *J. Amer. chem. Soc.* **72**, 3828.
Zaffaroni, A. (1953). *Recent Progr. Hormone Res.* **7**, 255.