reductase and NADPH₂ can be used to determine GSSG. The rate after the addition of cystamine gives total glutathione. However, GSH undergoes some oxidation during preparation of the extract and only total glutathione has been estimated in this study. The lower limit of the method as described is approx. 0.1 μ mole, and greater sensitivity can be achieved by using more glutathione reductase, anaerobic conditions and a more sensitive spectrophotometer. Since the cystamine concentration must approximately equal the GSH concentration a trial run is sometimes necessary.

The method described therefore is comparable in sensitivity and ease of manipulation with the glyoxalase method. The simple extraction procedure of heating, which works well for liver, muscle and brain, is, however, inapplicable for kidney, and with this tissue another method of extraction of GSH must be sought.

SUMMARY

1. The glyoxalase method for the estimation of glutathione has been adapted for use in the pH-stat.

2. A new method for the estimation of glutathione based on thiol-disulphide exchange reactions with cystamine and glutathione reductase has

been developed. The reactions are followed spectrophotometrically at 340 m μ .

3. The method as described can estimate down to 0.1 μ mole of glutathione.

4. It has been compared with the glyoxalase method and found to give similar results with rat liver, brain and muscle. The averages of these were 7.32, 1.90 and 1.1 μ moles/g. fresh wt. respectively.

5. Extracts of kidney tissue at pH 8.2, extracts of flour, and nicotinamide coenzymes form complexes with oxidized glutathione in such a way that it is unavailable for reduction by glutathione reductase.

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REFERENCES

- Conn, E. E. & Vennesland, B. (1951). *J. biol. Chem.* **192**, 17.
 Hird, F. J. R. (1962). *Biochem. J.* **85**, 320.
 Martin, H. & Mollwain, H. (1959). *Biochem. J.* **71**, 275.
 Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.
 Tallan, H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.
 Thompson, C. G. & Martin, H. (1959). *Symp. biochem. Soc.* **17**, 17.
 Woodward, G. E. (1935). *J. biol. Chem.* **109**, 1.

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Metabolism of 11-Oxygenated Steroids

3. SOME 1-DEHYDRO AND 9 α -FLUORO STEROIDS*

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In the two previous papers of this series (Bush & Mahesh, 1959*a*, *b*) it was shown that the reduction of orally administered 11-oxo steroids to the related 11 β -hydroxy steroids in man was greatly affected by the stereochemistry of the α -surface of the steroid molecule. In particular it was found that angulation of the A/B ring junction as in the 5 β -(H) steroids completely prevented this reduction, that a 2 α -methyl group greatly inhibited it, and that the partial angulation of the A/B ring

junction caused by the introduction of a 1,2-double bond into a Δ^4 -3-oxo steroid inhibited it appreciably (Bush & Mahesh, 1958*a*). These findings were taken to suggest that the 11-oxo steroid associated with the responsible enzyme ('steroid 11 β -ol dehydrogenase'; Hurlock & Talalay, 1959) over the 'upper' part of the α -surface of the steroid, i.e. with the closest apposition to the enzyme surface occurring with the α -oriented hydrogen atoms attached to C-1, C-2, C-12 and C-21 when present (Fig. 8 in Bush & Mahesh, 1959*b*). It was also possible to give a reasonable explanation of the increase in biological activity achieved by introducing a 2 α -methyl group into cortisol (11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione) and the paradoxical decrease achieved by a similar substitution

* Part 2: Bush & Mahesh (1959*b*).

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in cortisone (17 α ,21-dihydroxypregn-4-ene-3,11,20-trione) in terms of the effect of the 2 α -methyl group on the metabolism of the 11-oxygen function and of the Δ^4 -3-ketone group (see also Liddle, Richard & Tomkins, 1956; Glenn, Stafford, Lyster & Bowman, 1957; Peterson, Pierce, Wyngaarden, Bunim & Brodie, 1957).

In similar studies of the metabolism of orally administered 9 α -fluorocortisone and 9 α -fluorocortisol an entirely new set of phenomena were observed (Bush & Mahesh, 1958*a*), which called for a rather different explanation of the greatly increased biological activity obtained by substituting a 9 α -halogen in cortisone and cortisol (Fried & Sabo, 1954, 1957; Fried, 1957; Fried & Borman, 1958). The present paper describes our studies on the metabolism of 9 α -fluorocortisone, 9 α -fluorocortisol and their 1,2-dehydro derivatives in man. In order to consider the results obtained with 9 α -fluoroprednisone (1,2-dehydro-9 α -fluorocortisone) we include a full account of our previous results with prednisone (Bush & Mahesh, 1958*b*). Weinman Baulieu, Deltour & Jayle (1956) previously isolated 9 α -fluorocortisol from the urine of patients receiving 9 α -fluorocortisol 21-acetate.

METHODS AND MATERIALS

Methods

With the exception of specific items mentioned below, the methods were the same as those employed by Bush & Mahesh (1959*a, b*). A full account of the technical details of the estimation of steroids by the scanning of paper chromatograms is to be found in these papers and in Bush (1961, 1963). Briefly, the subjects were young males and were given the steroids as freshly crushed tablets or powders with a glass of milk between 9.0 and 9.30 a.m. They emptied their bladders immediately afterwards and collected all their urine for the next 24 hr. in a large glass bottle containing about 100 ml. of toluene.

The free and conjugated steroids were extracted from the urine by percolating it through a column of the anion-exchange resin Decolorite (The Permutit Co. Ltd., London) in the Cl⁻ form. After the column had been washed with water the adsorbed steroids were recovered by elution with methanol-aq. ammonia (sp.gr. 0.88) (19:1, v/v). This eluate was evaporated under reduced pressure and the residue dissolved in acetate buffer, pH 4.8. Succus entericus of *Helix pomatia* was added to give a concentration of β -glucuronidase of 1×10^3 – 2×10^3 units/ml., and the solution was incubated at 45° for 15–17 hr. The steroids were extracted with ether-ethyl acetate (2:1, v/v) and the extract was worked up in the usual way. The extract was then fractionated by chromatography on suitable widths of Whatman no. 3MM paper and three (or more) fractions were eluted for subsequent examination.

In each experiment a small part of each fraction was subjected to routine estimation by chromatography on Whatman no. 2 paper; the 17-oxo steroids in the two less polar fractions were estimated by treatment of the chro-

matograms with alkaline *m*-dinitrobenzene and scanning. With the most polar fraction, two chromatograms were run. One of them was treated with alkaline BT* and scanned, and the other was treated with NaOH and the yellow fluorescence of Δ^4 -3-oxo steroids was estimated visually by comparison with standards. Observer-bias was avoided in the latter method by coding the chromatograms (Bush & Mahesh, 1959*c*). After the zones revealed in these preliminary chromatograms had been fully identified, appropriate schemes of chromatograms for subfractionation were devised and used for the estimation of the individual steroids. These methods are described fully below. Samples of blood were drawn from a vein in the arm and analysed by methods described by Bush & Mahesh (1959*a, b*).

Chromatographic solvent systems

For convenience, these systems are referred to below by the following numbers: 1, benzene-methanol-water, (2:1:1, by vol.); 2, toluene-methanol-water (4:3:1, by vol.); 3, light petroleum-toluene-methanol-water (67:33:85:15, by vol.); 4, light petroleum-methanol-water (20:17:3, by vol.). Light petroleum was the fraction boiling between 100° and 120°.

Materials

Prednisone (17 α ,21-dihydroxypregna-1,4-diene-3,11,20-trione) (XXV, Scheme 4). This was used in the form of 5 mg. tablets (Meticorten) and was a gift from the Schering Corp., Bloomfield, N.J., U.S.A. Two tablets from each batch were pulverized and extracted with methanol-dichloromethane (1:1, v/v). A part of the extract sufficient for the detection of 1% (w/w) of any single impurity was run on a single-length chromatogram with solvent system 2 and the chromatograms were examined with ultraviolet light (at 2537 Å with a Chromatolite lamp; Hanovia Ltd., Slough, Bucks.) and by treatment with alkaline BT. No impurity was found and the material from the tablet gave a symmetrical zone of the same R_F as that of authentic prednisone.

9 α -Fluoroprednisone (9 α -fluoro-17 α ,21-dihydroxypregna-1,4-diene-3,11,20-trione) (XXXV, Scheme 5). This was in the form of 0.5 mg. tablets of the 21-acetate kindly given by Dr L. H. Sarett of Merck, Sharp and Dohme, Rahway, N.J., U.S.A. It was examined as above with solvent system 3 and found to be pure by the above criteria. The acetate of the related 11 β -ol [9 α -fluoroprednisolone (XXXIV, Scheme 5)] was a gift from the same source and found to be pure by the same methods.

9 α -Fluorocortisone (9 α -fluoro-17 α ,21-dihydroxypregn-4-ene-3,11,20-trione) (II, Scheme 1). This was prepared by oxidation of the 21-acetate of 9 α -fluorocortisol with chromium trioxide in aqueous acetic acid (Fried & Sabo, 1954) and hydrolysis with methanolic KHCO₃ overnight. The material was recrystallized from benzene-methanol and compared with an authentic sample (gift from Dr J. Fried, the Squibb Institute, New Brunswick, N.J., U.S.A.) by mixed melting point and chromatography in solvent system 1. It was pure by these criteria.

9 α -Fluorocortisol (9 α -fluoro-11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione) (I, Scheme 1). This was administered in the form of pure crystalline 9 α -fluorocortisol (a gift from Dr J. Fried). A similar gift from Dr J. A. Hogg of the Upjohn

* Abbreviation: BT, blue tetrazolium.

Co., Kalamazoo, Mich., U.S.A., and a gift of the 21-acetate from Dr L. H. Sarett (in tablet form) were also used. All three preparations were chromatographically pure when run in solvent systems 1 and 3.

Cortisol (11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione). A pure crystalline sample was used and was a gift from Dr J. A. Hogg. It was chromatographically pure in solvent system 2.

EXPERIMENTAL AND RESULTS

Metabolism of 9 α -fluorocortisone (Expt. A in Tables 1, 2 and 3)

A male subject aged 21 years took 25 mg. of 9 α -fluorocortisone (II) at 9:27 a.m. and collected all his urine for the next 24 hr. Samples of blood were drawn from his right antecubital vein 2, 88, 183, 268, 363 and 542 min. after the ingestion.

Identification of urinary steroids. A 750 ml. sample of urine (half the 24 hr. specimen) was extracted by percolation through a column of Decolorite (2.5 cm. $\times$ 15 cm.; bed volume 74 ml.). The column was washed with 100 ml. of water and the steroids were eluted with 500 ml. of methanol-aq. ammonia (sp.gr. 0.88) (19:1, v/v). The eluate was evaporated under reduced pressure for 15 min. at room temperature to remove most of the ammonia and then taken to near dryness on a water bath at 45°. The residue dissolved in buffer solution was treated with succus entericus of *Helix pomatia*, extracted and divided into three fractions by chromatography in solvent system 3 (single-length run).

After preliminary examination on single-length chromatograms, the three fractions were sub-fractionated as follows:

Fraction 1 (least polar steroids) was over-run for 14 hr. in solvent system 4 on Whatman no. 2 paper (25 cm. wide $\times$ 40 cm. long).

Fraction B (moderately polar steroids) was over-run for 14 hr. in solvent system 3 on Whatman no. 2 paper of the same size.

Fraction C (most polar steroids) was over-run for 20 hr. in solvent system 1 on Whatman no. 3MM paper of the same size.

The main components on the first two chromatograms were detected by treating strips 3–5 mm. wide with alkaline *m*-dinitrobenzene and heating, and those on the third chromatogram by treating with alkaline BT and by the fluorescence reaction with sodium hydroxide. These chromatograms and the subfractions that were eluted from them are shown in Fig. 1. The subfractions eluted from the first two chromatograms gave single zones when run again, but those from the third chromatogram, which was slightly overloaded, required further purification by running them again for 20 hr. in solvent system 1 on 25 cm. wide sheets of

Whatman no. 2 paper. Homogeneous subfractions were obtained by elution of these chromatograms.

During the subsequent application of sequential degradations to the subfractions many single zones gave two zones after acetylation. It soon became clear that, apart from the usual large separations of monoacetates from diacetates, several zones gave two acetates whose R_F values differed by the much smaller amount characteristic of epimeric 3 α -acetoxy-5 α -(H) and 3 α -acetoxy-5 β -(H) steroids (Bush & Willoughby, 1957). All the subfractions giving two acetates were therefore completely acetylated and subjected to further chromatographic separation and elution before proceeding with further degradations. The acetates of the substances found in fraction C were separated by over-run ($\times$ 2–4) chromatograms with solvent system 3; the acetates of the substances in fraction B were separated with solvent system 4 ($\times$ 1–2). The details are not given in the account below since the appropriate length of run can be inferred in each case from the R_F values given.

(a) Subfraction FE1. This material was very polar and remained at the origin after running for 20 hr. in solvent system 1. It gave a yellow fluorescence with sodium hydroxide and failed to reduce BT, and showed no change in these properties after oxidation with sodium bismuthate or chromic acid. The material gave no colour reaction with alkaline *m*-dinitrobenzene after these oxidations. Its identification was not pursued further.

(b) Subfraction FE2. The major portion gave a yellow fluorescence with sodium hydroxide and reduced BT weakly. Oxidation with sodium bismuthate gave a product that did not move from the origin after running for 3.5 hr. in solvent system 3 and that gave both a yellow fluorescence with sodium hydroxide and a bluish-purple colour with alkaline *m*-dinitrobenzene. Oxidation of this derivative with chromic acid gave a substance with R_F 0.11 in solvent system 3 and retaining the above colour reactions. The yields of these reactions were, however, low and identification could not be completed.

The minor product (III, Scheme 1) was separated from contaminants by acetylation, the acetate (IV) having R_F 0.08 in solvent system 3. It gave a yellow fluorescence with sodium hydroxide but did not reduce BT. The increase in R_F strongly suggested the presence of two esterifiable hydroxyl groups in the original substance. The latter (II) was therefore oxidized with sodium bismuthate to compound (V), which had R_F 0.18 in solvent system 3, and gave a yellow fluorescence with sodium hydroxide and a bluish-purple colour with alkaline *m*-dinitrobenzene. The same material (V) was obtained from compound (III) when periodic acid was used. The properties of compound (V)

were identical with those of 9 α -fluoro-11 β -hydroxyandrost-4-ene-3,17-dione (V) prepared by the oxidation of 9 α -fluorocortisol (I) with sodium bis-muthate. The properties of compound (V) were not

altered by acetylation at room temperature for 16 hr., but oxidation with chromic acid gave a new product with R_f 0.54 in solvent system 3 and which now gave, in addition to the yellow fluorescence

Fig. 1. Subfractionation of urinary extracts from Expts. A and B. Subfractions are coded as in the text. Fractions A, B and C were prepared as in Bush & Willoughby (1957). Conditions and solvent systems are indicated below each chromatogram. O, Origin; Z, solvent front. Fraction A (least polar): 1, dehydroepiandrosterone; 2, aetiocholanolone; 3, androsterone; O, colour with alkaline *m*-dinitrobenzene. Fraction B (moderately polar): 4, 11 β -hydroxyaetiocholanolone; 5, 11-oxoaetiocholanolone; O, colour with alkaline *m*-dinitrobenzene. Fraction C (most polar): 6, tetrahydrocortisol; 7, allotetrahydrocortisol; 8, tetrahydrocortisone; 9, 9 α -fluorocortisol; 10, 9 α -fluorocortisone; 11, cortisol; 12, cortisone; O, reducing BT; ●, yellow fluorescence with NaOH.

Scheme 1. Degradations used to characterize metabolites of 9 α -fluorocortisol (I) and 9 α -fluorocortisone (II) (Expts. A and B). Ac, CH₃·CO.

with sodium hydroxide, a pinkish colour, which appeared in the cold, with alkaline *m*-dinitrobenzene. These properties were identical with those of 9 α -fluoroandrost-4-ene-3,11,17-trione (VI) prepared by the oxidation of 9 α -fluorocortisone (II) with sodium bismuthate. The original minor component (III) of subfraction FE2 was therefore identified as 9 α -fluoro-11 β ,17 α ,20,21-tetrahydroxy-pregn-4-en-3-one.

Oxidation of the original mixed subfraction FE2 with sodium bismuthate gave small quantities of three other substances giving purple colour reactions with alkaline *m*-dinitrobenzene. One of these was 11 β -hydroxyandrost-4-ene-3,17-dione, and its precursor was identified as 20,20-dihydrocortisol (11 β ,17 α ,20,21-tetrahydroxypregn-4-en-3-one) by the sequence of degradations described previously (Chart 1 in Bush & Mahesh, 1959b). The other two products of oxidation with sodium bismuthate were identified as 9 α -fluoro-11 β -hydroxyandrostosterone (XX, Scheme 3) and 9 α -fluoro-11 β -hydroxyaetiocholanolone (IX, Scheme 2) by the sequence described for subfractions FE7 and FE8. It is reasonable to suppose that these were derived from 9 α -fluoro-3 α ,11 β ,17 α ,20,21-pentahydroxy-5 α - and 9 α -fluoro-3 α ,11 β ,17 α ,20,21-pentahydroxy-5 β -pregnane respectively, since the same derivatives were also obtained in similar yield by oxidation with periodic acid. The amounts, however, were too small to allow the isolation of the original compounds.

(c) Subfraction FE3. The material (VII, Scheme 2) in this fraction reduced alkaline BT and was more polar than tetrahydrocortisol (3 α ,11 β ,17 α ,21-

tetrahydroxy-5 β -pregnan-20-one). It gave no fluorescence with sodium hydroxide and on acetylation gave a reducing material (VIII) with R_F 0.54 in solvent system 3 (tetrahydrocortisol 3,21-diacetate has R_F 0.56). Oxidation of compound (VII) itself with sodium bismuthate gave a product (IX) having R_F 0.07 in solvent system 3 (11 β -hydroxyaetiocholanolone has R_F 0.11) and giving a bluish-purple colour with alkaline *m*-dinitrobenzene on heating. Acetylation of compound (IX) gave a material (X) with R_F 0.36 in solvent system 4 (11 β -hydroxyaetiocholanolone 3-acetate has R_F 0.45) and giving the same colour reaction with alkaline *m*-dinitrobenzene. Oxidation of compound (X) with chromic acid gave a new product (XI) with R_F 0.57 in solvent system 4 (11-oxoaetiocholanolone 3-acetate has R_F 0.54). Treatment of compound (XI) with cold sodium hydroxide overnight (Bush & Willoughby, 1957) gave a new product (XII) with R_F 0.33 in solvent system 3 (11-oxoaetiocholanolone has R_F 0.26). Compounds (XI) and (XII) both gave a pink reaction with alkaline *m*-dinitrobenzene in the cold similar to that given by 11,16-dioxo steroids. Finally, oxidation of compound (XII) with chromic acid gave a new substance (XIII) with R_F 0.69 in solvent system 3 (aetiocholanone-3,11,17-trione has R_F 0.57). This substance (XIII) gave an immediate pink colour with alkaline *m*-dinitrobenzene which became bluish-purple on heating, typical of 3,11,17-trioxo steroids.

The chromatographic behaviour of the products of acetylation (VIII, X and XI) was only com-

patible with the configuration 5 β -(H)-3 α -hydroxyl (Bush & Willoughby, 1957; Bush, 1961). The very large change in R_F on oxidation of the unprotected hydroxyl group with chromic acid is characteristic of the 9 α -fluoro-11 β -hydroxyl function (e.g. X $\rightarrow$ XI). Compound (XIII) was identified as 9 α -fluoroaetiocholan-3,11,17-trione by preparing it on the microgram scale (mixed with the 5 α -epimer, XV) from compound (I) by the route shown in Scheme 2 and found to have properties identical with those of the above derivative. Finally, the fluorine atom was removed from compound (XII) by refluxing it with collidine for 15 min. (Fried & Sabo, 1957). The product (XIV) had R_F 0.17 in

solvent system 3 and, unlike compound (XII), showed absorption in the ultraviolet with a peak at 240 m μ (due to the $\Delta^{9(10)}$ -11-one structure).

The original material (VII) in subfraction FE3 was therefore identified as 9 α -fluorotetrahydrocortisol (9 α -fluoro-3 α ,11 β ,17 α ,21-tetrahydroxy-5 β -pregnan-20-one).

(d) Subfraction FE4. This material (XVI, Scheme 3) reduced alkaline BT but gave no fluorescence with sodium hydroxide. It had the same R_F as tetrahydrocortisol. On acetylation two substances were obtained, one with the R_F of tetrahydrocortisol 3,21-diacetate, the other (XVII) with a lower R_F (in solvent system 3). These two sub-

Scheme 2. Degradations used to characterize 5 β -(H) ring-A-saturated metabolites of 9 α -fluorocortisol (I) and 9 α -fluorocortisone (II) (Scheme 1). Ac, CH₃·CO.

stances were separated and then subjected to a similar sequence of degradations as with subfraction FE3 by using derivatives prepared from 9 α -fluorocortisol as reference substances.

Comparison of the diacetates of compounds (VII) and (XVI) immediately suggested that compound (XVI) was the allo [i.e. 5 α -(H)] epimer of compound (VII). The partial saponification of compound (XVII) to compound (XVIII) (Bush & Willoughby, 1957) was therefore carried out and compound (XIX) was obtained by oxidation with bismuthate for an accurate comparison of its R_F

with that of compound (X) (Scheme 2). Since the chromatographic behaviour of both compound (XVII) and compound (XIX) compared with compounds (VIII) and (X) was characteristic of that of epimeric 3 α -acetoxy-5 β -(H) and 3 α -acetoxy-5 α -(H) steroids, and the terminal oxidation products (XIII and XV) were indistinguishable triones (Bush & Willoughby, 1957), compound (XVI) was identified as 9 α -fluoroallotetrahydrocortisol (9 α -fluoro-3 α ,11 β ,17 α ,21-tetrahydroxy-5 α -pregnan-20-one).

(e) Subfraction FE5. This subfraction contained

Scheme 3. Degradations used to characterize 5 α -(H) ring-A-saturated metabolites of 9 α -fluorocortisol (I) and 9 α -fluorocortisone (II) (Scheme 1). Compound (XV) $\rightarrow$ compound (XXIII) was carried out on synthetic material as a check on the synthesis of compound (XV), but not on compound (XV) obtained from urinary compound (XVI). Ac, $\text{CH}_3\cdot\text{CO}$.

small amounts of allotetrahydrocortisol and traces of tetrahydrocortisone, which were identified by the sequence of degradations described previously (Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*). A trace of 9 α -fluorocortisol (I) was also found.

(*f*) Subfraction FE6. This material had the same R_f as tetrahydrocortisone in solvent system 1, and both reduced alkaline BT and gave a strong fluorescence with sodium hydroxide. On acetylation two reducing substances were found on chromatograms with solvent system 3; one had the same R_f as tetrahydrocortisone 3,21-diacetate, the other the same R_f as 9 α -fluorocortisol (I, Scheme 1). Only the latter material gave a yellow fluorescence with sodium hydroxide. The former was saponified (Bush & Willoughby, 1957) and identified as tetrahydrocortisone 3,21-diacetate by the sequence given in Fig. 1 of Bush & Mahesh (1959*a*).

The presumed 9 α -fluorocortisol (I) was identified as such by oxidation with bismuthate to compound (V), which has R_f 0.18 in solvent system 3, giving a yellow fluorescence with sodium hydroxide and a bluish-purple colour with alkaline *m*-dinitrobenzene. These properties were identical with those of 9 α -fluoro-11 β -hydroxyandrost-4-ene-3,17-dione prepared by the same route from 9 α -fluorocortisol. Compound (V) was unchanged by acetylation at room temperature for 16 hr., but on oxidation with chromic acid gave a substance (VI) with R_f 0.54 in solvent system 3, and giving a yellow fluorescence with sodium hydroxide and a pink-purple colour reaction, which began in the cold, with alkaline *m*-dinitrobenzene. These properties were identical with those of 9 α -fluoroadrenosterone (VI) prepared from 9 α -fluorocortisol (I) by the same route. They are typical of 9 α -fluoro-11-oxygenated steroids in that the R_f of the 11 β -ol is smaller and that of the 11-ketone larger than those of the related steroids lacking a 9 α -fluorine atom (Bush, 1961).

(*g*) Subfraction FE7. This contained a substance which did not give a yellow fluorescence with sodium hydroxide and which gave a bluish-purple colour with alkaline *m*-dinitrobenzene. It was identified as 9 α -fluoro-11 β -hydroxyaetiocholanolone (IX, Scheme 2) by the sequence used in the identification of 9 α -fluorotetrahydrocortisol (VIII, Scheme 2).

(*h*) Subfraction FE8. This material gave a bluish-purple colour with alkaline *m*-dinitrobenzene, and on acetylation gave two substances giving the same reactions. They gave no fluorescence with sodium hydroxide and their R_f values in solvent system 4 differed by an amount characteristic of 3 α -hydroxy-5 β -(H) and 3 α -hydroxy-5 α -(H) steroids after acetylation (Bush & Willoughby, 1957; Bush, 1961). One of them was identified as

11 β -hydroxyaetiocholanolone by the sequences used by Bush & Mahesh (1959*a*); the other was identified as 9 α -fluoro-11 β -hydroxyandrosterone (XX, Scheme 3) by the terminal reactions (substances XIX, XXI, XXII, XV, XXIII) used to identify 9 α -fluoroallotetrahydrocortisol (XVI, Scheme 3).

(*i*) Subfraction FE9. This contained 11 β -hydroxyandrosterone, which was identified by the sequence used by Bush & Mahesh (1959*a*).

(*j*) Subfraction FE10. This contained 11-oxo-aetiocholanolone, which was identified by the sequence used by Bush & Mahesh (1959*a*).

(*k*) Subfraction FE11. This contained a substance giving a pinkish-purple colour reaction, which began in the cold, with alkaline *m*-dinitrobenzene. It had the same properties as 9 α -fluoro-11-oxo-aetiocholanolone (XII, Scheme 2) obtained in the sequence employed with 9 α -fluorotetrahydrocortisol (VII), and was identified as such by completing the sequence to obtain compound (XIV).

(*l*) Subfractions FE12 and FE13. These contained aetiocholanolone and androsterone respectively, which were identified by their reaction with alkaline *m*-dinitrobenzene, their R_f values, and those of their acetates (Bush & Willoughby, 1957).

Quantitative estimation of metabolites (Tables 1 and 2). One-hundredth of the 24 hr. specimen of urine was extracted by the resin method and run for 16 hr. in solvent system 1 by using the central 2.0 cm. of strips of Whatman no. 2 paper 2.5 cm. wide. The zones previously identified as 20,20-dihydrocortisol, 9 α -fluoro-20,20-dihydrocortisol (III) and 9 α -fluorocortisol (I) were estimated visually against standards by the fluorescence reaction with sodium hydroxide.

One-twentieth of the 24 hr. specimen was extracted by the same method and subfractionated in the routine way. A part of each fraction was subjected to our routine method as follows (Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*).

Fraction A: One-fifth ($\equiv 0.01 \times 24$ hr. urine); 14 hr. run in solvent system 4; scanned after treatment with alkaline *m*-dinitrobenzene.

Fraction B: One-half ($\equiv 0.025 \times 24$ hr. urine); 14 hr. run in solvent system 3; scanned after treatment with alkaline *m*-dinitrobenzene.

Fraction C: One-fifth ($\equiv 0.01 \times 24$ hr. urine); 14–16 hr. run in solvent system 1; scanned after treatment with alkaline BT.

To resolve the substances confused on these standard chromatograms, one-fifth of fraction C and one-half of fraction B were then acetylated and run for 4 hr. in solvent system 3 and for 5 hr. in solvent system 4 respectively. These chromatograms were then treated with alkaline *m*-dinitrobenzene (fraction B) and alkaline BT (fraction C)

and scanned in the usual way. Because of the characteristic inversion of the relative mobilities of 3 α -hydroxy steroids epimeric at C-5 (Bush & Willoughby, 1957) it was possible in this way to achieve an estimation of all the metabolites in this type of mixture (see Figs. 2 and 3).

Estimation of steroids in plasma. A 5 ml. sample of plasma was used for each estimation. The extracts were run for 6–8 hr. in solvent system 2 after concentrating the original deposit to a very small spot at the origin of the chromatogram (Bush & Mahesh, 1959c). The results are shown in Table 3. At 1.5 hr. after the administration of 9 α -fluorocortisone the smallest approximate concentrations (μ g./100 ml.) detectable with this volume of plasma would have been: cortisol, 0.4 μ g.; 9 α -fluorocortisol, 0.2 μ g.; cortisone, 0.8 μ g.; 9 β -fluorocortisone, 0.5 μ g. Therefore the 9 α -fluorocortisol:9 α -fluorocortisone concentration ratio must have been at least 10:1 for 9 α -fluorocortisone to have escaped detection.

Metabolism of 9 α -fluorocortisol (Expt. B in Tables 1, 4 and 5)

A male subject aged 30 years took 25 mg. of 9 α -fluorocortisol at 9.17 a.m. He collected his urine for the next 24 hr., and blood samples were drawn at 0, 90, 185, 270, 365 and 540 min. after the ingestion.

The metabolites observed were the same as in the experiment with 9 α -fluorocortisone except that 9 α -fluoro-11-oxo-aetiocholanolone (XII) was not detected. Identification and estimation of the metabolites was carried out in the same way as in Expt. A and the results are given in Tables 1 and 4. The results of the estimation of the steroids in plasma are given in Table 5.

Metabolism of prednisone (Expt. C in Tables 1 and 6)

A male subject 21 years of age took 5 mg. of prednisone by mouth at 8.00 a.m., 1.0 p.m. and

Table 1. *Excretion of normal steroid metabolites in Expts. A–D*

Experimental details are given in the text. The results are expressed as mg./24 hr. corrected for losses as described by Bush & Mahesh (1959a, b). In Expt. C the subject had taken prednisone (5 mg. three times daily) for the 3 days preceding the experiment.

Precursor given ...	Expt. A 9 α -Fluorocortisone (25 mg.)	Expt. B 9 α -Fluorocortisol (25 mg.)	Expt. C Prednisone (50 mg.)	Expt. D 9 α -Fluoroprednisone 21-acetate (25 mg.)
Steroids excreted				
Tetrahydrocortisol	0.43	0.30	1.4	0.93
Tetrahydrocortisone	0.52	0.32	1.1	1.2
Allotetrahydrocortisol	1.8	1.1	1.0	0.56
Cortisol	0.0*	0.0	0.0	0.0
Cortisone	0.0†	0.0	0.0	0.0
11 β -Hydroxyaetiocholanolone	0.06	0.06	0.25	0.06
11-Oxo-aetiocholanolone	0.39	0.16	0.15	0.0
11 β -Hydroxyandrosterone	0.26	0.20	0.25	0.12
Dehydroepiandrosterone	0.0‡	0.0	0.0	0.0
Aetiocholanolone	2.0	2.6	2.2	2.0
Androsterone	2.8	3.0	2.4	1.8

* < 0.015 mg./24 hr.

† < 0.030 mg./24 hr.

‡ < 0.10 mg./24 hr.

Table 2. *Excretion of metabolites of 9 α -fluorocortisone (Expt. A)*

Experimental details are given in the text. The results are corrected as described by Bush & Mahesh (1959a, b).

Steroid metabolite	Excretion	
	(mg./24 hr.)	(% of dose)
9 α -Fluoro-3 α ,11 β ,17 α ,20,21-pentahydroxy-5 β -pregnane	0.10	0.40
9 α -Fluoro-3 α ,11 β ,17 α ,20,21-pentahydroxy-5 α -pregnane	0.10	0.40
9 α -Fluorotetrahydrocortisol	1.3	5.0
9 α -Fluoroallotetrahydrocortisol	2.1	8.2
9 α -Fluoro-20,20-dihydrocortisol	0.12	0.48
9 α -Fluorocortisol	0.26	1.0
9 α -Fluoro-11 β -hydroxyaetiocholanolone	0.30	1.2
9 α -Fluoro-11 β -hydroxyandrosterone	0.56	2.2
9 α -Fluoro-11-oxo-aetiocholanolone	0.14	0.56
Total (not rounded) ...	4.98	19.44

8.00 p.m. for 3 days. On the fourth day he took 50 mg. of prednisone by mouth at 9.00 a.m., collected his urine until 9.00 a.m. the next day, and had blood drawn at 12 noon.

Identification of urinary steroids. One-fifth of the 24 hr. sample was extracted and the usual fractions were obtained from a single-length chromatogram in solvent system 3. The most polar fraction (fraction 3) was subfractionated by running for 16 hr. in solvent system 1 on Whatman no. 2 paper; fraction 2 was subfractionated similarly in solvent system 3 with a 14 hr. run; fraction 1 (least polar) was subfractionated by a 14 hr. run in solvent system 4.

The principal metabolites were detected by their absorption of ultraviolet light (at 2537 Å with a Hanovia Chromatolite lamp), reduction of BT, fluorescence with sodium hydroxide, or colour reactions with alkaline *m*-dinitrobenzene. The normal metabolites of endogenous origin were identified by the sequences of degradations described by Bush & Mahesh (1959*a, b*) and estimated by scanning in the usual way. The following metabolites of prednisone were identified (Scheme 4).

(a) Fraction 3 (most polar). The subfraction more polar than tetrahydrocortisol was run in solvent system 1 for 20 hr. on Whatman no. 2 paper. Two

Fig. 2. Typical scanning records (BT) showing method of estimating mixed zones containing C_{21} metabolites of cortisol (endogenous) and 9 α -fluorocortisone or 9 α -fluorocortisol (administered). (a) Chromatogram of fraction C from Expt. A: 1, 9 α -fluorotetrahydrocortisol; 2, tetrahydrocortisol mixed with 9 α -fluoroallotetrahydrocortisol; 3, allotetrahydrocortisol; 4, tetrahydrocortisone mixed with 9 α -fluorocortisol. (b) Chromatogram of same fraction C after acetylation: 5, 9 α -fluorocortisol 21-acetate; 6, 9 α -fluoroallotetrahydrocortisol 3,21-diacetate; 7, 9 α -fluoro-tetrahydrocortisol 3,21-diacetate mixed with allotetrahydrocortisol 3,21-diacetate; 8, tetrahydrocortisol 3,21-diacetate; 9, tetrahydrocortisone 3,21-diacetate. The six individual compounds were estimated as 1 and 3 on chromatogram (a) (as free steroids) and as 5, 6, 8 and 9 on chromatogram (b) (as acetates).

Fig. 3. Scanning records (alkaline *m*-dinitrobenzene) showing estimation of mixed zones of C_{19} metabolites of cortisol and 11 β -hydroxyandrost-4-ene-3,17-dione (endogenous) and 9 α -fluorocortisone or 9 α -fluorocortisol (administered). (a) Chromatogram of fraction B from Expt. B: 1, 9 α -fluoro-11 β -hydroxyaetiocholanolone; 2, 11 β -hydroxyaetiocholanolone mixed with 9 α -fluoro-11 β -hydroxyandrost-4-ene-3,17-dione; 3, 11 β -hydroxyandrost-4-ene-3,17-dione; 4, 11-oxoaetiocholanolone. (b) Chromatogram of same fraction B after acetylation: 5, 9 α -fluoro-11 β -hydroxyandrost-4-ene-3,17-dione 3-acetate; 6, 9 α -fluoro-11 β -hydroxyaetiocholanolone 3-acetate mixed with 11 β -hydroxyandrost-4-ene-3,17-dione 3-acetate; 7, 11 β -hydroxyaetiocholanolone 3-acetate; 8, 11-oxoaetiocholanolone 3-acetate. The five individual compounds were estimated as 1, 3 and 4 on chromatogram (a) (as free steroids) and as 5, 6 and 7 on chromatogram (b) (as acetates). In this case, measurement of 8 gives an internal control on the recovery of 4 from the acetylation procedure.

Table 3. Plasma steroids after the oral administration of 9 α -fluorocortisone (Expt. A)

Experimental details are given in the text. The results are expressed as $\mu\text{g./100 ml.}$ and are corrected for losses as described by Bush & Mahesh (1959*a, b*). See the text for the significance of zero values.

Time after dose	...	Concn. in plasma				
		0 hr.	1.5 hr.	3.1 hr.	4.5 hr.	9.0 hr.
Cortisol		9.0*	0.0	2.0	0.0	0.0
9 α -Fluorocortisol		0.0	5.0	1.0	1.0	0.0
9 α -Fluorocortisone		0.0	0.0	0.0	0.0	0.0

* Normally in the range 5–20 $\mu\text{g./100 ml.}$

substances were observed by their absorption of ultraviolet light, one 3.4 cm. and the other 9.1 cm. from the origin. They both gave the combined periodate-Zimmermann reaction (Bush, 1955). Tetrahydrocortisol moved 9.7 cm. from the origin on the same sheet. Neither compound reduced BT, nor did they give a yellow fluorescence with sodium hydroxide. The more polar of the two substances (XXXI) was oxidized by both periodic acid and sodium bismuthate to an apparently single substance (XXVIII) with R_f 0.15 in solvent system 3 and which absorbed ultraviolet light and gave a bluish-purple colour with alkaline *m*-dinitrobenzene on heating. The properties of this material were unchanged by acetylation at room temperature, but oxidation with chromic acid gave a new substance (XXIX) with R_f 0.35 in solvent system 3. This material absorbed ultraviolet light, gave no fluorescence with sodium hydroxide, and gave a pinkish-purple colour reaction, which began in the cold, with alkaline *m*-dinitrobenzene. The properties of compound (XXVIII) and (XXIX) were identical with those of 11 β -hydroxyandrosta-1,4-diene-3,17-dione and of androsta-1,4-diene-3,11,17-trione (Scheme 4) run on the same sheet. These steroids had been prepared by the oxidation of prednisolone (XXIV) with sodium bismuthate to compound (XXVIII), and then with chromic acid to compound (XXIX). The more polar compound (XXXI) was therefore identified as 20,20-dihydro-

prednisolone (11 β ,17 α ,20,21-tetrahydroxypregna-1,4-diene-3-one), discovered as a metabolite of prednisone by Gray, Green, Holness & Lunnon (1956).

The less polar substance (XXX) was oxidized by both periodic acid and by sodium bismuthate to a product (XXIX) having the properties of androsta-1,4-diene-3,11,17-trione in these tests. It was unchanged by acetylation and by oxidation with chromic acid. Compound (XXX) was therefore identified as 20,20-dihydroprednisone (17 α ,20,21-trihydroxypregna-1,4-diene-3,11-dione), described by Gray *et al.* (1956).

A third material in fraction 3 moved 10.3 cm. from the origin when run for 16 hr. in solvent system 1 and both reduced BT and absorbed ultraviolet light. On acetylation, two substances reducing BT were found. One of these absorbed ultraviolet light and had the same R_f (0.01) as prednisolone acetate (XXVI) in solvent system 3; the other did not absorb ultraviolet light and had the same R_f as allotetrahydrocortisol diacetate (Bush & Willoughby, 1957) in solvent system 3. After hydrolysis of compound (XXV) with methanolic bicarbonate the former substance (XXIV) was obtained and was oxidized with sodium bismuthate to give compound (XXVIII), identified as 11 β -hydroxyandrosta-1,4-diene-3,17-dione as above.

A fourth substance in fraction 3 absorbed ultraviolet light, reduced BT, and had the same R_f as

Table 4. *Excretion of metabolites of 9 α -fluorocortisol (Expt. B)*

Experimental details are given in the text. The results are corrected as described by Bush & Mahesh (1959*a*, *b*).

Steroid metabolite	Excretion	
	(mg./24 hr.)	(% of dose)
9 α -Fluoro-3 α ,11 β ,17 α ,20,21-pentahydroxy-5 β -pregnane	0.10	0.40
9 α -Fluoro-3 α ,11 β ,17 α ,20,21-pentahydroxy-5 α -pregnane	0.10	0.40
9 α -Fluorotetrahydrocortisol	1.0	4.0
9 α -Fluoroallotetrahydrocortisol	2.0	8.0
9 α -Fluoro-2,20-dihydrocortisol	0.12	0.48
9 α -Fluorocortisol	0.25	1.0
9 α -Fluoro-11 β -hydroxyaetiocholanolone	0.32	1.3
9 α -Fluoro-11 β -hydroxyandrosterone	0.50	2.0
Total (not rounded)	4.39	17.58

Table 5. *Plasma steroids after the oral administration of 9 α -fluorocortisol (Expt. B)*

Experimental details are given in the text. The results are expressed as μ g./100 ml. and are corrected for losses as described by Bush & Mahesh (1959*a*, *b*). See the text for the significance of zero values.

Time after dose ...	0 hr.	1.5 hr.	3.0 hr.	4.5 hr.	9.0 hr.
Cortisol	7.0*	—†	0.0	0.0	0.0
9 α -Fluorocortisol	0.0	—†	0.5	7.0	1.0
9 α -Fluorocortisone	0.0	—†	0.0	0.0	0.0

* Normally in the range 5–20 μ g. 100/ml.

† Sample lost.

prednisone (XXV) in solvent systems 1 and 2. On acetylation, it gave a product with R_f 0.07 in solvent system 3 and having the same properties as prednisone 21-acetate (XXVII). This material was apparently unaltered by oxidation with chromic acid. After hydrolysis with methanolic bicarbonate to the original substance (XXV), oxidation with sodium bismuthate gave compound (XXIX), identified as androsta-1,4-diene-3,11,17-trione as above.

(b) Fraction 2. This fraction contained two substances that both absorbed ultraviolet light and gave purple colour reactions with alkaline *m*-dini-

trobenzene. The more polar substance was apparently unchanged by acetylation, moved 16.1 cm. in a 14 hr. run in solvent system 3, and was oxidized with chromic acid to a material that ran 34 cm. under the same conditions. The less polar substance ran 34 cm. in solvent system 3 and was apparently unchanged by acetylation at room temperature or by oxidation with chromic acid. These properties were identical with those of 11 β -hydroxyandrosta-1,4-diene-3,17-dione (XXVIII) and androsta-1,4-diene-3,11,17-trione (XXIX) run on the same sheets. The two metabolites were therefore identified with these two substances,

Scheme 4. Degradations used to characterize metabolites of prednisone (XXV) (Expt. C). Ac, CH₃·CO.

which had been described as metabolites of prednisone by Gray *et al.* (1956).

Quantitative estimation of metabolites (Table 6). The two 20,20-dihydro metabolites (XXX and XXI) of prednisone were estimated by taking the subfraction more polar than tetrahydrocortisol, oxidizing it with sodium bismuthate, acetylating it at room temperature to remove contaminating 3-hydroxy steroids and running the products for 14 hr. in solvent system 3. After treatment with alkaline *m*-dinitrobenzene, the chromatograms were scanned. Appropriate standards were run by carrying out a similar procedure with prednisolone and prednisone in known quantities, thus controlling any losses involved in the chemical transformation (Bush & Mahesh, 1959b).

Prednisolone and prednisone were estimated by acetylating a known amount of fraction 3 and running the products for 14 hr. in solvent system 3. The chromatograms were treated with alkaline BT and scanned together with appropriate standards put through the same process to control losses.

Steroids in plasma. A 12 ml. sample of plasma was extracted and the extracts were deposited and concentrated at the origin of chromatograms in the usual way for plasma steroids. To detect prednisone and prednisolone, however, the chromatograms, after being run for 8 hr. in solvent system 2, were treated with alkaline BT as for the scanning for reducing steroids. Because of the very small quantities, they were estimated by visual comparison with standards of 0.1, 0.2, 0.5, 1.0 and 2.0 μg . of cortisol, prednisone and prednisolone. The sample of blood collected just before the administration of the 50 mg. dose of prednisone contained no detectable reducing substances. The sample collected 3 hr. after the dose contained two substances with the same mobilities as those of prednisone and prednisolone and which were identified as such. The amounts were estimated as 0.9 μg . of prednisone and 1.0 μg . of prednisolone: the difference is insignificant by this method.

Metabolism of 9 α -fluoroprednisone (Expt. D in Tables 1 and 7)

9 α -Fluoroprednisone 21-acetate (25 mg.) was taken by a male subject aged 24 years at 10.30 a.m. He collected a 24 hr. sample of urine, and venous blood was drawn at 1.30 p.m.

Identification of metabolites in the urine (Scheme 5). The urine was extracted and fractionated on preliminary chromatograms as in the experiment with prednisone. We confined our attention to the polar C_{21} metabolites since we were mainly concerned with the state of the 11-oxygen function and preliminary chromatograms showed that the expected C_{19} metabolites were not detectable.

In the subfraction more polar than tetrahydrocortisol, one substance (XXXII) absorbing ultraviolet light was detected which gave a purple colour with the combined periodate-Zimmermann reaction. This substance moved 2.7 cm. in a 20 hr. run in solvent system 1, whereas 20,20-dihydroprednisolone (XXXI, Scheme 4), identified in the previous experiment, moved 3.4 cm. It did not reduce alkaline BT, and on oxidation with either periodic acid or sodium bismuthate gave rise to a product (XXXVIII) with R_f 0.12 in solvent system 3, and which absorbed ultraviolet light and gave a bluish-purple colour with alkaline *m*-dinitrobenzene on heating. These properties were unchanged by acetylation at room temperature, but oxidation with chromic acid gave a new product (XXXIX) with R_f 0.35 in solvent system 3 and which now gave a pink-purple colour reaction, which began in the cold, with alkaline *m*-dinitrobenzene. The properties of compounds (XXXVIII) and (XXXIX) were identical with those of 9 α -fluoro-11 β -hydroxyandrost-1,4-diene-3,17-dione and of 9 α -fluoroandrost-1,4-diene-3,11,17-trione respectively, prepared by the oxidation of 9 α -fluoroprednisolone with sodium bismuthate to compound (XXXVIII), and subsequent oxidation to compound (XXXIX) with chromic acid. The original material (XXXII) was therefore identified

Table 6. *Excretion of metabolites of prednisone (Expt. C)*

Experimental details are given in the text. The results are corrected as described by Bush & Mahesh (1959*a, b*).

Steroid metabolite	Excretion	
	(mg./24hr.)	(% of dose)
20,20-Dihydroprednisolone	0.09	0.18
20,20-Dihydroprednisone	0.08	0.16
Prednisolone	0.76	1.5
Prednisone	0.77	1.5
11 β -Hydroxyandrost-1,4-diene-3,17-dione	0.030	0.060
Androst-1,4-diene-3,11,17-trione	0.026	0.052
Total (not rounded)	1.756	3.452

as 20,20-dihydro-9 α -fluoroprednisolone (9 α -fluoro-11 β ,17 α ,20,21-tetrahydroypregna-1,4-dien-3-one). The related 11-ketone (XXXIII) was not detected.

On the preliminary chromatograms of fraction 3 it was found that the zone occupied by presumed tetrahydrocortisol absorbed ultraviolet light and had moved the same distance as 9 α -fluoroprednisolone (XXXIV) itself (8.1 cm.) in a 16 hr. run in solvent system 1 on Whatman no. 2 paper. The material from this zone was acetylated at room temperature and run for 17 hr. on Whatman no. 2 paper in solvent system 3. An apparently homo-

geneous substance (XXXVI) was found 4.0 cm. from the origin and this absorbed ultraviolet light and reduced alkaline BT. These properties were identical with those of 9 α -fluoroprednisolone 21-acetate run on the same sheet.

Oxidation of the original subfraction with sodium bismuthate gave two products that were detected on a single-length chromatogram in solvent system 3. One was identified, by its colour reaction, failure to absorb ultraviolet light, R_f , and the chromatographic properties of its acetate, as 11 β -hydroxyaetiocholanolone (Fig. 1 in Bush &

Scheme 5. Degradations used to characterize metabolites of 9 α -fluoroprednisone (XXXV) (Expt. D). Ac, CH₃·CO.

Table 7. *Excretion of metabolites of 9 α -fluoroprednisone 21-acetate (Expt. D)*

Experimental details are given in the text. The results are corrected as described by Bush & Mahesh (1959*a, b*).

Steroid metabolite	Excretion	
	(mg./24 hr.)	(% of dose)
9 α -Fluoro-20,20-dihydroprednisolone	0.10	0.40
9 α -Fluoroprednisolone	1.2	4.8
9 α -Fluoroprednisone	0.12	0.48
Total (not rounded) 	1.42	5.68

Mahesh, 1959*a*), and was derived from the tetrahydrocortisol in the original subfraction. The other (in slightly greater quantity) was identified as 9 α -fluoro-11 β -hydroxyandrost-1,4-diene-3,17-dione (XXXVIII) by the procedure described above. The original ultraviolet-absorbing material in this subfraction was therefore identified as 9 α -fluoroprednisolone (XXXIV).

A third substance found in fraction 3 had the same mobility as 9 α -fluoroprednisone (XXXV) in solvent system 1 (14.6 cm. in 16 hr. on Whatman no. 2 paper), absorbed ultraviolet light and reduced alkaline BT. Acetylation at room temperature gave an apparently homogeneous product (XXXVII) that moved the same distance as 9 α -fluoroprednisone 21-acetate (8.4 cm.) on the same sheet in a 17 hr. run in solvent system 3 on Whatman no. 2 paper. Oxidation of compound (XXXV) with sodium bismuthate gave a product (XXXIX) identified as 9 α -fluoroandrost-1,4-diene-3,11,17-trione by the procedure given above.

Estimation of urinary steroids. These were carried out by the same procedure as in Expt. C, namely by oxidation of the subfraction more polar than tetrahydrocortisol with bismuthate to the related 17-oxo steroids, and by acetylation of the rest of fraction 3. The results are given in Table 7.

Estimation of steroids in plasma. This was carried out exactly as in Expt. C with 25 ml. of plasma collected 3 hr. after the administration of the steroid. A reducing substance with the same mobility as 9 α -fluoroprednisolone was found and the quantity was estimated visually as 1.1 μ g. No 9 α -fluoroprednisone was detected, although 0.10 μ g. of this steroid was just detectable under the same conditions.

DISCUSSION

Reliability of the methods employed. We have previously given a general discussion of the validity of our methods (Bush & Mahesh, 1959*a, b*; Bush, 1961) and only the points peculiar to the present experiments are discussed below. The present work was done before the application of the R_M treatment to the chromatographic behaviour of steroids had been carried out (Brooks, Hunt, Long &

Mooney, 1957; Bush, 1960; Kabasakalian & Basch, 1960), so that the chromatographic properties of the metabolites and their derivatives has been considered descriptively. In the first place, we emphasize that all the degradations were carried out under conditions giving high yields; in each case a degradation of a known steroid was carried out in parallel with the unknown, and the yield of both reactions ascertained approximately by careful visual comparison with standards (95 % confidence limits about ± 15 % under our conditions). The quantities used were always sufficient to allow the detection of more than 10 % of any single impurity and usually enough to detect 5 % (i.e. within that class of substances detected by the method used). Finally, it was found valuable to observe all the available colour and spectroscopic properties of each derivative and compare them carefully with known quantities of appropriate standards. Thus, for instance, in the identification of 9 α -fluoroprednisolone both its reduction of alkaline BT and its absorption of ultraviolet light on the chromatogram were compared with standards and an approximate estimate of quantity was made by each method. It was possible immediately to suggest that the original subfraction contained a mixture of this steroid and tetrahydrocortisol because the estimated value for the reduction of BT was nearly twice that for the ultraviolet absorption. When the two compounds had been separated as their acetates, the quantity of the presumed 9 α -fluoroprednisolone 21-acetate was estimated to be approximately the same by both methods, the estimate by ultraviolet absorption being the same as with the original subfraction. This agreement was maintained after oxidation of the subfraction with sodium bismuthate, when estimates were made from the ultraviolet absorption and the reaction with alkaline *m*-dinitrobenzene. Many pitfalls are avoided by adopting this procedure as a general rule (Bush, 1961).

Losses during extraction were assessed as described in previous papers of this series, namely by using the correlation we had observed between the recoveries of known steroids in our procedure and their partition coefficients in the hydrocarbon-

aqueous methanol solvent systems used for chromatography (Bush & Mahesh, 1959a). Losses during estimations which involved one or more chemical transformations were controlled by subjecting similar amounts of a similar or identical reference steroid to all the steps of the procedure in parallel with the fraction (Bush & Mahesh, 1959b).

Though more satisfactory results would have been obtained by using precursors labelled with radioactive isotopes, or by the isolation of larger amounts of material, this was not feasible for various reasons. Labelled precursors were not available and the amounts of precursors that were available were small; again, all but one of the precursors (prednisone) are extremely potent steroids and their administration for any length of time, even if it had been possible, is not without actual or potential risks to health. Finally, we were anxious to examine the metabolism of these substances in doses that did not raise their concentration in plasma much above the normal range of concentrations of cortisol in human plasma.

Metabolism of the 11-oxygen function. Our main objective was to study the extent to which the 11-oxo group of the three 11-oxo steroid precursors was reduced to an 11 β -hydroxyl group after oral administration. By far the most striking result was the total absence of all but one of the expected 11-oxo steroid metabolites of 9 α -fluorocortisone from the urine of the subject taking this steroid, although the 11 β -hydroxy metabolites analogous to those of cortisone itself were all found to be present. When 9 α -fluorocortisol was given, it was impossible to detect any of the expected 11-oxo steroid metabolites. Despite the finding that considerable changes in the 11-oxo steroid:11 β -hydroxy steroid metabolites concentration ratio in human urine can occur under conditions of extreme physical trauma (Gold, Singleton, MacFarlane & Moore, 1958), there is a large body of evidence showing that the naturally secreted 11 β -hydroxy steroid hormones are excreted predominantly as the related 11-oxo steroids; there is no reported case of a human being under normal conditions excreting 11 β -hydroxy steroids in the absence of any 11-oxo steroids (Bush, 1962a). This, coupled

with the fact that the subject of Expt. A excreted a significant amount of the normal 11-oxo steroid metabolites of cortisol (Table 1), strongly supports the conclusion that an individual anomaly of metabolism was not the cause of this unusual finding.

The same effect of the 9 α -fluorine atom was seen in comparing the metabolism of prednisone with that of 9 α -fluoroprednisone (Expts. C and D). As reported by Bush & Mahesh (1958b, 1959b), prednisone (11-ketone) appears to be reduced to prednisolone (11 β -ol) more slowly than cortisone; this is most clearly shown by the fact that the plasma contains roughly equal quantities of prednisolone and prednisone shortly after an oral dose of prednisone (Expt. C), whereas the cortisol:cortisone concentration ratio in plasma after the oral administration of cortisone is normally in the range 10–15:1 (Bush & Mahesh, 1959b). Approximately the same ratios are found in the urinary concentrations of these four compounds under these conditions (Bush & Mahesh, 1959b; Table 6 in the present paper). By contrast, the 9 α -fluoroprednisolone:9 α -fluoroprednisone concentration ratio after the administration of the 11-ketone was $\geq 11:1$ in the plasma and 10:1 in the urine (Expt. D in Table 7). The effect of the 9 α -fluorine atom does not completely counteract the opposite effect of the 1,2-double bond. The relevant ratios are summarized in Table 8.

Other aspects of the metabolism of 9 α -fluoro steroids. The percentage recovery of recognizable metabolites from these precursors was lower than with similar experiments with steroids lacking both the 9 α -fluorine atom and the 1,2-double bond of prednisone and its derivatives. With 9 α -fluorocortisone and 9 α -fluorocortisol the excretion of metabolites amounts to just under half the total excretion of the analogous metabolites of cortisone that would be expected under similar conditions (Bush & Mahesh, 1959a; Fukushima, Bradlow, Hellman, Zumoff & Gallagher, 1960). On the other hand, a fairly large amount of material giving the yellow fluorescence with sodium hydroxide that is characteristic of Δ^4 -3-oxo steroids and of few other substances (Bush, 1954; Neher, 1959) was found on chromatograms of the urinary

Table 8. Plasma and urine 11 β -ol:11-ketone concentration ratios after the administration of the related 11-ketone

Experimental details are given in the text. The values for cortisone are from Bush & Mahesh (1959b).

Precursor	Related 11 β -ol	[11 β -ol]:[11-ketone] ratio	
		In plasma	In urine
Cortisone	Cortisol	≥ 15	14
Prednisone	Prednisolone	1	1
9 α -Fluorocortisone	9 α -Fluorocortisol	≥ 10	≥ 50
9 α -Fluoroprednisone	9 α -Fluoroprednisolone	≥ 11	10

extracts from these experiments (A and B) and could not be identified. This material was extremely polar so that the recovery during the extraction of the urine was probably low. Though it probably contained at least some 2 α - and 6 β -hydroxylated metabolites of the 9 α -fluoro steroids (Burstein, Dorfman & Nadel, 1954; Bush & Mahesh, 1959*b*), it appeared to be much more labile than similar metabolites of other precursors.

The percentage recovery of prednisone and 9 α -fluoroprednisone as recognized metabolites was even lower (3.5 and 5.7 % respectively). Our search for unusual metabolites was, however, not exhaustive, since our main aim was to examine the 11 β -hydroxy:11-oxo ratio of the precursor and its principal metabolites in plasma and urine. We would, however, have detected any significant amounts of metabolites in our extracts that possessed groups reducing alkaline BT, the dihydroxyacetone side chain, the 17-ketone group, the $\Delta^{1,4,3}$ -ketone group, or the glycerol side chain. A probable explanation of these low recoveries is that these rather polar precursors are less effectively absorbed from the alimentary tract in man (Schedl & Clifton, 1961).

Vermeulen (1959) obtained 98 % recovery of injected [^{14}C]prednisolone as radioactivity in a subject receiving 80 μg . of unlabelled prednisolone by mouth, and absorption of the orally administered steroid seemed to be 80–100 % complete in this subject. This, however, was during the last 2 days of a 4-day period of oral administration, and would be expected to show a larger percentage recovery than in our experiments where the loading dose was only 15 mg./day, prednisone was used instead of prednisolone and urine was collected for 1 day only. The pattern of metabolites observed by Vermeulen (1959) was similar to that described above, although his values were based on the isolation of material by crystallization and his recoveries by this method were low. Apart from individual variations, Schedl & Clifton (1961) have shown that

the alimentary absorption of prednisone and its more heavily substituted derivatives is relatively inefficient in man.

One other feature of the metabolism of these precursors was observed, namely the unusually large proportion of 5 α -(H) steroids among the metabolites of 9 α -fluorocortisone and 9 α -fluorocortisol. The values for analogous metabolites of these two steroids and of cortisone and cortisol are summarized in Table 9. This has also been found with three other subjects given 9 α -fluorocortisol not reported here. Also, the pattern of the metabolites of endogenously secreted cortisol was altered in the same direction (Table 1). Thus the allotetrahydrocortisol [5 α -(H)]:tetrahydrocortisol [5 β -(H)] ratio in the urines of subjects A and B were 4.2 and 3.7 respectively, and the 11 β -hydroxyandrosterone:11 β -hydroxyaetiocholanolone ratios were similarly large. This again is unlikely to be due to individual anomalies of metabolism, since no single case of this ratio exceeding 0.7 has been described (Bush & Willoughby, 1957; Romanoff, Seelye, Rodriguez & Pincus, 1957), and its mean value in 52 subjects both with and without adrenocortical dysfunction studied by the former authors was 0.38. In other experiments, the subjects of these studies have been found to excrete these steroids in normal ratios of 5 α -(H) and 5 β -(H) steroids.

Significance of the results. Various other studies have been made of the metabolism and distribution of some of these steroids. A large number of studies have shown that 1,2-dehydro steroids such as prednisone are eliminated from the blood much more slowly than cortisol (e.g. Ely, Done & Kelley, 1956; Sandberg & Slaunwhite, 1957) and metabolized to inactive products more slowly than cortisol by preparations of rat livers *in vitro* (e.g. Schriefers, Korus & Dirscherl, 1957; Glenn, 1959). Again, it has been shown that many steroid analogues of this type associate less strongly with transcortin than does cortisol itself (Slaunwhite & Sandberg, 1959; Silber, 1959). The metabolism of

Table 9. 5 α -(H) Metabolites:5 β -(H) metabolites concentration ratios of various Δ^4 -3-ketones

Experimental details are given in the text. The values for cortisone alone are from Bush & Mahesh (1959*b*).

Precursor	[5 α -(H) Metabolites]:[5 β -(H) metabolites] ratio	
	Total	Total
	C ₂₁ metabolites	C ₁₉ metabolites
Cortisone (alone)	0.23	0.18
Cortisone (in the presence of 9 α -fluorocortisone: Expt. A)	1.9	0.57
Cortisone (in the presence of 9 α -fluorocortisol: Expt. B)	1.8	0.91
9 α -Fluorocortisone (Expt. A)	1.6	1.3
9 α -Fluorocortisol (Expt. B)	2.0	1.6

prednisone has been investigated in some detail by Gray *et al.* (1956) and by Caspi & Pechet (1957, 1958). The main conclusion of this earlier work was that much of the increase in the biological activity of such analogues of cortisone and cortisol was due to the decreased rate at which they were inactivated by hepatic enzyme systems. The question of the redox reaction at C-11 undergone by such steroids was not closely investigated by these authors, although inspection of some of the results of Schriefers *et al.* (1957) suggests that some of the peculiarities we have observed in our experiments can also be seen in theirs.

In the two previous papers of this series (Bush & Mahesh, 1959*a, b*) we gave reasons for believing that our results were largely or entirely due to the substrate-specificity of the hepatic enzymes responsible for the metabolism of these steroids and not to secondary factors such as differences in the mode of absorption or distribution of these steroids. The same argument holds for the compounds studied in the present paper, although the low yields of identifiable metabolites make it impossible to exclude the existence of other pathways of metabolism for these analogues of cortisone. However, our interpretations of the earlier and of the present experiments has been confirmed in almost every detail (see, however, Meigs & Engel, 1961) by experiments *in vitro* with homogenates, microsomes and acetone-dried powders of rat livers (Bush & Hunter, 1961, 1962; Bush, Meigs & Hunter, 1962). The experiments *in vitro*, however, add one facet that could not have been predicted with confidence from the results *in vivo*. We concluded from the results *in vivo* that the introduction of a 9 α -fluorine atom increased the rate of reduction of 11-oxo steroids by the responsible enzyme system (Expts. A and D), and that 9 α -fluorocortisol might actually prevent or greatly inhibit the rate of oxidation by the system of related 11 β -hydroxy steroids. The results of experiments *in vitro* show clearly that both these interpretations are correct (Bush *et al.* 1962; I. E. Bush & S. A. Hunter, unpublished work).

It seems likely that the main effect of the 9 α -fluorine atom on the oxidoreduction of 11-oxygen functions in steroids is due to its negative inductive effect on the 11-oxygen function, and not to any large effect on the association of the steroid with the enzyme, or on the mechanism of the enzyme reaction itself. Thus A. Eschenmoser (personal communication) has found that the 9 α -fluorine atom produces a considerable decrease in the rate at which 11 β -hydroxy steroids are oxidized to the 11-ketones by chromic acid. From a thermodynamic analysis of his results, he concludes that this is due to the greatly increased stability of the hydroxyl group owing to the negative inductive

effect of the 9 α -fluorine atom. It would be expected therefore that, in the absence of any influence of the fluorine atom on the enzyme reaction itself, the enzymic reduction of 9 α -fluoro-11-ketones would be increased in rate, and that, with the enzyme system operating *in vivo* under equilibrium or steady-state conditions, the metabolites of either 9 α -fluoro-11-ketones or of 9 α -fluoro-11 β -ols would be made up of a larger proportion of 11 β -ols than with 11-oxygenated steroids lacking the 9 α -fluorine atom. The almost complete absence of the expected 11-ketones in our experiments, however, in the presence of all the expected and related 11 β -ols, may require an additional factor for its complete explanation, namely that, as suggested above, the 9 α -fluoro-11 β -hydroxy steroids actually inhibit competitively the oxidative direction of this redox reaction at C-11.

The biological significance of these results has been discussed by Bush (1962*a, b*), and is only dealt with briefly below. Fried (1957) first suggested that the greatly increased biological activity of the 9 α -fluoro analogues of natural corticosteroids was due to an increased acidity of the 11 β -hydroxyl group. This would strengthen a hydrogen bond between the 11 β -hydroxyl group and a proton-accepting group in a receptor molecule for the hormone, but would weaken such a bond if the receptor formed it by means of proton-donation (Bush, 1962*b*). It is very likely, however, that the greatly increased stability of the reduced form of such steroids (i.e. the 11 β -ol) also plays a considerable part in enhancing the biological activity of the 9 α -fluoro corticosteroid analogues. Thus the ratio of the biological activities of 9 α -fluorocortisol and 9 α -fluorocortisone is 1.0, whereas the ratio in other similar pairs of natural steroids is 2.0 in favour of the reduced form (Bush, 1962*b*). This would be expected on the basis of the present view that the 11-ketone form of such steroids is not itself effective at the receptors for glucocorticoids. Thus the exceptionally rapid reduction of the 9 α -fluoro-11-ketone and the stability of the resultant 11 β -ol would prevent the 'wastage' due to oxidation to the 11-ketone that occurs with naturally occurring corticosteroids. The 'protection' of the 11 β -hydroxyl group by 9 α -halogen atoms was first suggested as a possible reason for their enhanced biological activity by Callow, Lloyd & Long (1954) in connexion with 9 α -chlorocortisol.

SUMMARY

1. The metabolism of 9 α -fluorocortisol, 9 α -fluorocortisone, prednisone and 9 α -fluoroprednisone 21-acetate was investigated after their oral administration to young male subjects, with

special reference to the oxidation-reduction of the 11-oxygen function of these steroids. The excretion of the metabolites of endogenously secreted steroids was also measured during the experiments, and the concentrations of cortisol and of the administered analogues in the plasma were measured at the same time.

2. Both the 9 α -fluoro-11-ketones gave rise to a greater proportion of the related 11 β -ols in the plasma, shortly after administration, than was found with the halogen-free parent steroids.

3. 9 α -Fluorocortisol and 9 α -fluorocortisone gave rise to a set of metabolites analogous to those given by cortisol and cortisone as far as reduction of the A-ring structures, and reduction and removal of the side chain were concerned. The most striking finding, however, was that the expected 11-ketones were almost completely absent from the urine of the subject given 9 α -fluorocortisone, and undetectable in the urine of the subject given 9 α -fluorocortisol. A similar difference was found between the metabolites of 9 α -fluoroprednisone 21-acetate and those of prednisone itself.

4. The ring-A-saturated metabolites of 9 α -fluorocortisol and 9 α -fluorocortisone included a far greater proportion of 5 α -(H) steroids than has been found with the halogen-free parent steroids. Similarly, the metabolism of endogenous cortisol was altered in the same direction during the experiments, resulting in considerably increased relative amounts of allotetrahydrocortisol and 11 β -hydroxyandrosterone in the urine.

5. It is suggested that these results can be explained in terms of two probable mechanisms. First, the negative inductive effect of the fluorine atom causes an increase in the stability of the 11 β -ol relative to the 11-ketone resulting in a purely thermodynamic effect increasing the rate of reduction and decreasing the rate of oxidation in the redox reaction 11 β -ol $\rightleftharpoons$ 11-ketone. Secondly, it seems probable that certain 9 α -fluoro-11 β -hydroxy steroids inhibit the oxidative direction of the reaction and may also selectively inhibit 5 β -reduction of the 4,5-double bond in Δ^4 -3-oxo steroids.

6. A comparison with results obtained *in vitro* suggests that these results are largely or entirely due to differences in the hepatic transformation of these steroids and not to other factors such as their mode of absorption or distribution.

7. The results add further confirmation to earlier suggestions that the biological actions of 11-oxygenated steroids depend on the interaction of the 11 β -hydroxy form with tissue receptors and not on the redox reaction at C-11 itself, except in so far as the latter indirectly affects the proportion of 11 β -ol and 11-ketone that is available to the tissues.

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REFERENCES

- Brooks, S. G., Hunt, J. S., Long, A. G. & Mooney, B. (1957). *J. chem. Soc.* p. 1175.
- Burstein, S., Dorfman, R. I. & Nadel, E. M. (1954). *Arch. Biochem. Biophys.* **53**, 307.
- Bush, I. E. (1954). *Recent Progr. Hormone Res.* **9**, 321.
- Bush, I. E. (1955). *Biochem. J.* **59**, 14P.
- Bush, I. E. (1960). *Symp. biochem. Soc.* **18**, 1.
- Bush, I. E. (1961). *The Chromatography of Steroids*. Oxford: Pergamon Press Ltd.
- Bush, I. E. (1962a). *Pharmacol. Rev.* **14**, 317.
- Bush, I. E. (1962b). *The Human Adrenal Cortex*, p. 138. Ed. by Currie, A. R., Symington, T. & Grant, J. K. Edinburgh: E. and S. Livingstone Ltd.
- Bush, I. E. (1963). *Meth. biochem. Anal.* **11**, 149.
- Bush, I. E. & Hunter, S. A. (1961). *Biochem. Pharmacol.* **8**, 142.
- Bush, I. E. & Hunter, S. A. (1962). *Biochem. J.* **84**, 16P.
- Bush, I. E. & Mahesh, V. B. (1958a). *Biochem. J.* **69**, 9P.
- Bush, I. E. & Mahesh, V. B. (1958b). *Biochem. J.* **69**, 21P.
- Bush, I. E. & Mahesh, V. B. (1959a). *Biochem. J.* **71**, 705.
- Bush, I. E. & Mahesh, V. B. (1959b). *Biochem. J.* **71**, 718.
- Bush, I. E. & Mahesh, V. B. (1959c). *J. Endocrin.* **18**, 1.
- Bush, I. E., Meigs, R. A. & Hunter, S. A. (1962). *J. Endocrin.* **24**, ii.
- Bush, I. E. & Willoughby, M. L. N. (1957). *Biochem. J.* **67**, 689.
- Callow, R. K., Lloyd, J. & Long, D. A. (1954). *Lancet*, ii, 20.
- Caspi, E. & Pechet, M. M. (1957). *Arch. Biochem. Biophys.* **68**, 236.
- Caspi, E. & Pechet, M. M. (1958). *J. biol. Chem.* **230**, 843.
- Ely, R. S., Done, A. K. & Kelley, V. C. (1956). *Proc. Soc. exp. Biol., N.Y.*, **91**, 503.
- Fried, J. (1957). *Cancer*, **10**, 752.
- Fried, J. & Borman, A. (1958). *Vitam. & Horm.* **16**, 303.
- Fried, J. & Sabo, E. (1954). *J. Amer. chem. Soc.* **76**, 1455.
- Fried, J. & Sabo, E. (1957). *J. Amer. chem. Soc.* **79**, 1130.
- Fukushima, D. K., Bradlow, H. L., Hellman, L., Zumoff, B. & Gallagher, T. F. (1960). *J. biol. Chem.* **235**, 2246.
- Glenn, E. M. (1959). *Endocrinology*, **64**, 373.
- Glenn, E. M., Stafford, R. O., Lyster, S. C. & Bowman, B. J. (1957). *Endocrinology*, **61**, 128.
- Gold, N. I., Singleton, E., MacFarlane, D. A. & Moore, F. D. (1958). *J. clin. Invest.* **37**, 813.
- Gray, C. H., Green, M. A. S., Holness, N. J. & Lunnon, J. B. (1956). *J. Endocrin.* **14**, 146.

- Hurlock, B. & Talalay, P. (1959). *Arch. Biochem. Biophys.* **80**, 468.
- Kabasakalian, P. & Basch, A. (1960). *Analyt. Chem.* **32**, 458.
- Liddle, G. W., Richard, J. E. & Tomkins, G. M. (1956). *Metabolism*, **5**, 384.
- Meigs, R. A. & Engel, L. L. (1961). *Endocrinology*, **69**, 152.
- Neher, R. (1959). *Chromat. Rev.* **1**, 99.
- Peterson, R. E., Pierce, C. E., Wyngaarden, J. B., Bunim, J. J. & Brodie, B. B. (1957). *J. clin. Invest.* **36**, 1301.
- Romanoff, L. P., Seelye, J., Rodriguez, R. & Pincus, G. (1957). *J. clin. Endocrin. Metab.* **17**, 434.
- Sandberg, A. A. & Slaunwhite, W. R. (1957). *J. clin. Endocrin. Metab.* **17**, 1040.
- Schedl, H. P. & Clifton, J. A. (1961). *Gastroenterology*, **41**, 491.
- Schriebers, H., Korus, W. & Dirscherl, W. (1957). *Acta endocr., Kbh.*, **26**, 331.
- Silber, R. H. (1959). *Ann. N.Y. Acad. Sci.* **82**, 821.
- Slaunwhite, W. R. & Sandberg, A. A. (1959). *J. clin. Invest.* **38**, 384.
- Vermeulen, A. (1959). *J. Endocrin.* **18**, 278.
- Weinmann, S., Baulieu, E. E., Deltour, G. & Jayle, M. F. (1956). *C.R. Acad. Sci., Paris*, **242**, 197.

Biochem. J. (1964), **93**, 255

Aspects of Calcification

THE AVAILABILITY OF ϵ -AMINO GROUPS IN COLLAGEN AGGREGATES

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Soluble collagen of connective tissue can be precipitated as six different types of fibril each representing a specific mode of aggregation of rod-like collagen molecules. Five of the reconstituted collagen aggregates are interconvertible, namely the 640 Å period, 220 Å period and structureless fibrils, the fibrous long-spacing fibril and the segment long-spacing aggregate, yet only the 640 Å fibril is found in Nature (for review see Schmitt, 1956). A nodular reconstituted collagen fibril has also been described (Kahn, Carrol & Witnauer, 1961).

Neuman & Neuman (1953) were the first to suggest that the mineralization of bone and dentine was initiated by collagen fibrils and their epitactic theory of calcification (Neuman & Neuman, 1958) has been further developed by Glimcher and his co-workers.

Glimcher, Hodge & Schmitt (1957) showed that the 640 Å fibril was the only collagen aggregate capable of nucleating hydroxyapatite (bone salt) crystals *in vitro* from metastable solutions (Strates, Neuman & Levinskas, 1957) of calcium phosphate.

By blocking the ϵ -amino groups of native collagen with FDNB*, Glimcher (1960) has established that these phosphate-binding groups are essential for calcification *in vitro*.

Solomons & Irving (1958) have demonstrated, however, that the ϵ -amino groups of soft-tissue collagen are less accessible to FDNB than those of bone or dentine. The unnatural collagen fibrils might, therefore, be prevented from calcifying *in*

vitro by a lower accessibility of their ϵ -amino groups rather than an absence of the unique stereostructure of the 640 Å fibril. The present work was designed to investigate this possibility.

Collagen aggregates were dinitrophenylated by the procedure of Levy & Li (1955), a method discussed by Fraenkel-Conrat, Harris & Levy (1955). It was thought that, by causing the aggregates to react in purely aqueous media under simulated physiological conditions, the experimental results could be more closely related to those of Glimcher *et al.* (1957) than would be possible had the conventional Sanger (1945) dinitrophenylation procedure been employed.

EXPERIMENTAL

Materials

Acetic acid-soluble calf-skin collagen. Purified calf dermis (10 g.) was extracted with 0.5 N-acetic acid (600 ml.) for 24 hr. at 4° with occasional gentle stirring (Wood & Keech, 1960). The viscous collagen extract was strained through muslin and the residue of highly swollen insoluble collagen re-extracted (24 hr.) with sufficient 0.5 N-acetic acid to give a total volume of about 600 ml. After being strained through muslin, the combined extracts were clarified by filtration through coarse, medium and fine sintered glass.

A portion (500 ml.) of the acid filtrate was dialysed against three changes of 10 l. of 0.17 M-NaCl for 3 days at 4°. After being adjusted to pH 7.4 with 0.1 N-NaOH, the dialysed solution was incubated at 37° for 1 hr. The rigid collagen gel was spun down at 2500g, washed with water until free from Cl⁻ ions and freeze-dried. A portion (500 mg.) of the product was dissolved in 500 ml. of

* Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.