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Isolation and Characterization of Human Urinary Metabolites of Aldosterone. III. Three Isomeric Tetrahydro Metabolites

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After the oral administration of a large quantity of tritium-labeled aldosterone to normal human subjects, three isomeric urinary metabolites reduced in ring A were isolated and characterized. These compounds were 3α,11β,21-trihydroxy-18-oxoallopregnan-20-one; 3β,11β,21-trihydroxy-18-oxopregnan-20-one, and 3α,11β,21-trihydroxy-18-oxopregnan-20-one.

The isolation and characterization of human urinary metabolites of aldosterone has been the subject of previous reports from this laboratory (Ulick and Lieberman, 1957; Kelly *et al.*, 1962). This report concerns the isolation and proof of structure of two new tetrahydrometabolites from the urine of subjects who had ingested a large quantity of H^3 - *d* - aldosterone - 21 - monoacetate. These compounds are 3α,11β,21-trihydroxy-18-oxoallopregnan - 20 - one (3α,5α - tetrahydroaldosterone, M10) and 3β,11β,21 - trihydroxy-18-oxopregnan - 20 - one (3β,5β - tetrahydroaldosterone, M11). The chemical reactions by which they were characterized are shown in Figure 1.

Ulick and Lieberman (1957) isolated the major metabolite of aldosterone and tentatively assigned to it the structure 3α,18,21-trihydroxypregnane-11,20-dione. Recently, Ulick *et al.* (1961, and in preparation) determined that as originally isolated this metabolite was a mixture of 3α,18,21-trihydroxypregnane - 11,20 - dione (18 - hydroxy-THA) and 3α,11β,21 - trihydroxy - 18 - oxopregnan - 20 - one (3α,5β - tetrahydroaldosterone, M12), and that only the latter is a metabolite of aldosterone. In the present study the correct structure proposed by Ulick *et al.* (1961, and in preparation) was confirmed, and the metabolite was further characterized through the preparation of several new derivatives as shown in Figure 2.

Experimental

Melting points were determined on a Kofler block and were corrected. Infrared spectra were obtained on a Perkin-Elmer Model 221 spectrometer. A Packard Liquid Scintillation Spectrometer was used for the assay of radioactivity, and simultaneous counting of tritium and C^{14} was done according to the discriminator ratio method of Okita *et al.* (1957). The determination of the number of acetylatable hydroxyl groups and the partition chromatography were carried out as previously described (Kelly *et al.*, 1962). The chromatography systems employed are listed in Table I.

H^3-Aldosterone-21-monoacetate.—One mg of *d*-aldosterone-21-monoacetate was randomly labeled with tritium in the laboratory of the New England Nuclear Corporation by exposure to tritium gas according to the procedure of Wilzbach (1957), and the labile tritium was exchanged for hydrogen by equilibration with methanol. The sample was returned to this laboratory for purification. About 20 mg of carrier was added to the radioactive material and three crystallizations were carried out, during which there was a small decrease in specific activity of the crystals. However, the specific activities of the mother liquor were much greater than those of the crystals, and increased with successive crystallizations. After the third crystallization the specific activity of the mother liquor was more than twice that of the crystals. The mother liquors were combined for further purification. The crystals were chromatographed in system D on a 50 g column, and the specific activity of each radioactive fraction was determined with the blue tetrazolium reaction (Recknagel and Litteria, 1956) used to estimate the weight of the aldosterone acetate. The mean specific activity of the fractions comprising the peak was $10.9 \times 10^6 \pm 1.0 \times 10^6$ cpm/mg. The fractions were combined and crystallized twice. The final crystals weighed 2.598 mg and had a specific activity of 10.6×10^6 cpm/mg. To this

TABLE I
PARTITION SYSTEMS EMPLOYED IN COLUMN CHROMATOGRAPHY

System	Components	g Celite/ml Stat. Phase
A	Ethyl acetate 1.2, ligroin 0.8, methanol 0.5, water 0.5	1.33
B	Benzene 4, methanol 2, water 1	2
C	Methylene chloride 0.75, cyclohexane 0.25, ethylene glycol 0.1	2
D	Isooctane 0.5, *t*-butanol 0.25, water 0.45, methanol 0.1	2
E	Ethyl acetate 0.40, hexane 0.60, methanol 0.35, water 0.15	2
G	Ethyl acetate 1, hexane 1, methanol 0.7, water 0.3	1
O	Ethyl acetate 6, toluene 4, formamide 1	2
P	Isooctane 0.6, *t*-butanol 0.4, methanol 0.1, water 0.35.	2
Q	Heptane 1, methanol 0.9, water 0.1	2
R	Ethyl acetate 0.2, ligroin B 0.8, methanol 0.7, water 0.3	2
S	Ligroin C 1.0, methanol 0.85, water 0.15	2
T	Isooctane 1.0, methanol 0.95, water 0.05	2
U	Benzene 1, ligroin 3, methanol 2, water 1	2
V	Benzene 0.76, ligroin 1.24, methanol 1.0, water 0.5	2
E_4[a]	Isooctane 0.5, *t*-butanol 0.225, water 0.05, methyl alcohol 0.225	2

[a] Eberlein and Bongiovanni (1955).

sample was added 98.7 mg of carrier, to give a calculated specific activity of 2.86×10^5 cpm/mg. Subsequent crystallizations yielded crystals of specific activity 3.11 and 2.78×10^5 cpm/mg and mother liquors of 2.62 and 3.18×10^5 cpm/mg respectively. The mean specific activity of the crystals and the mother liquor was $2.84 \times 10^5 \pm 0.14 \times 10^5$ cpm/mg.

All of the pure material was combined and diluted with carrier to a specific activity of about 1.70×10^5 cpm/mg, then crystallized, with no change in specific activity, in order to insure complete mixing of carrier and labeled aldosterone-21-monoacetate. The material was designated batch I.

All impure fractions containing tritiated aldosterone-21-monoacetate, including the combined mother liquors of the first three crystallizations, were combined and chromatographed in system D. The combined fractions were diluted with carrier to give a specific activity of 9.00×10^5 cpm/mg. A series of crystallizations revealed a small increase in specific activity of the mother liquors. Therefore the sample was chromatographed in system G. The combined fractions from the chromatogram had a specific activity of 8.50×10^5 cpm/mg. This material was diluted to 535 mg, for which a specific activity of 1.34×10^5 cpm/mg was calculated. Upon crystallization,

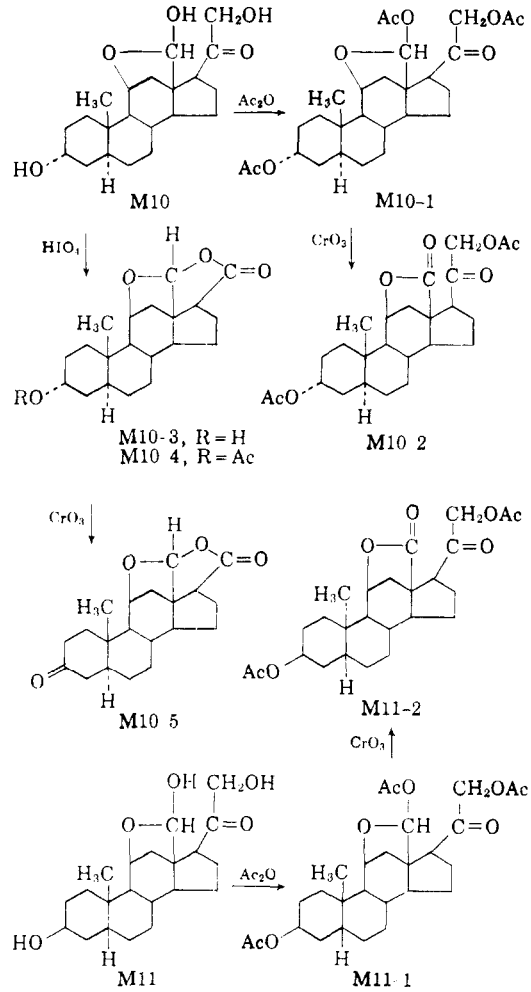

FIG. 1.—Flow sheet for the characterization of M10 and M11. The (18 → 11) hemiacetal form of the metabolites is shown in this figure and Figure 2 because they react as if they exist predominantly in this form. The structures shown are not meant to exclude the possibility of equilibrium among the open form and the two possible stereoisomeric hemiacetals, *cf.* Results and Discussion.

this material yielded a first crop with specific activity of 1.33×10^5 cpm/mg, a second crop of specific activity 1.30×10^5 cpm/mg, and a mother liquor of specific activity 1.31×10^5 cpm/mg. This sample (batch II) was administered to the subjects together with batch I. The composite specific activity of the administered labeled aldosterone was 1.40×10^5 cpm/mg, and this specific activity was expected for the metabolites.

The Administration of H^3-Aldosterone

The labeled hormone was administered orally to 11 normal young volunteers (4 females, 7 males) in a dose of 10 mg every 12 hours until 50 to 80 mg was taken. The dose of radioactivity was about 15 μc for each subject. In this way, a total of 760 mg was administered. All of the

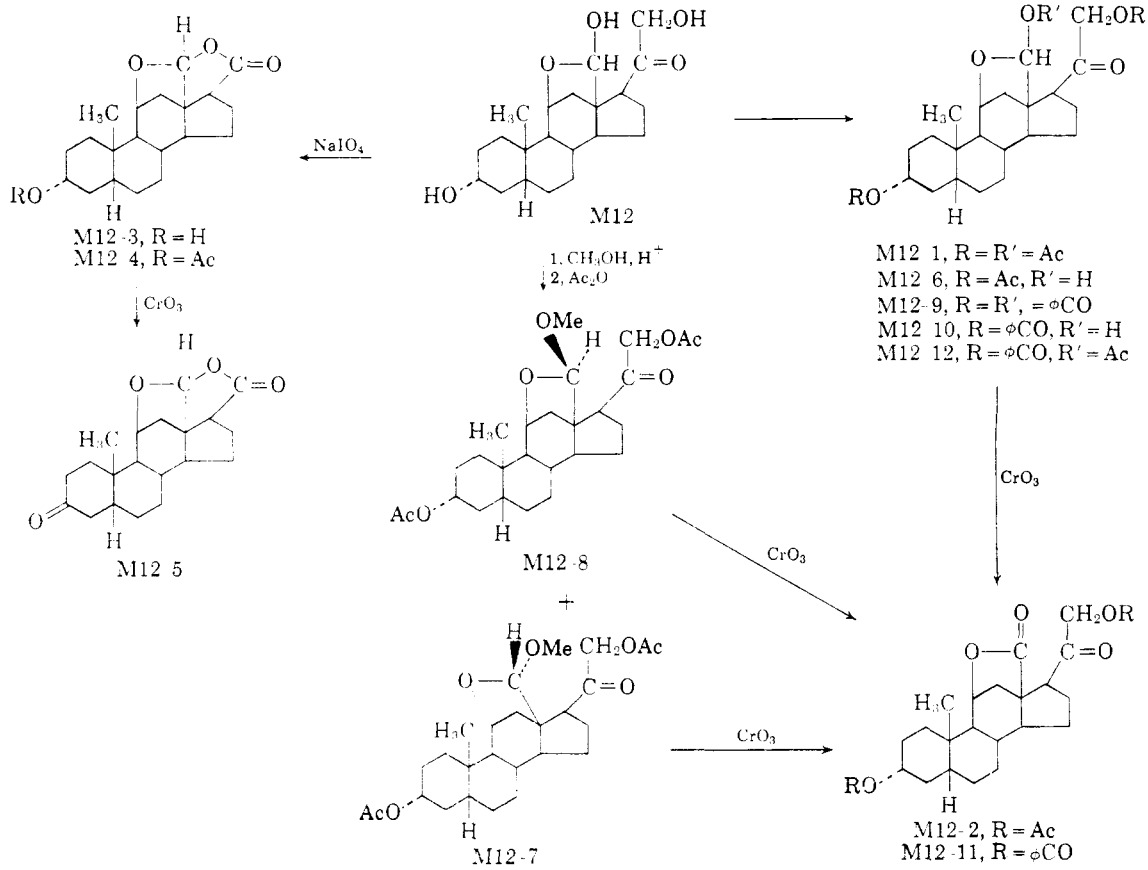

FIG. 2.—Flow sheet for the characterization of M12.

urine was collected during the period of administration and for at least 36 hours thereafter. No special precautions were taken, and no effects which could be attributed to the hormone were observed in any of the subjects. A total of 70 liters of urine was collected and pooled for isolation of metabolites of aldosterone.

Isolation of Metabolites of Aldosterone

Hydrolysis and Extraction of the Urine

The urine was hydrolyzed with β-glucuronidase (Ketodase, Warner-Chilcott Co.) and extracted with ethyl acetate as previously described (Kelly *et al.*, 1962). After extraction, the aqueous phase was adjusted to *p*H 1, saturated with NaCl, and extracted in five equal fractions with 70 liters of freshly distilled tetrahydrofuran. The tetrahydrofuran layer was washed with saturated $NaHCO_3$ solution. The washes were diluted and adjusted to *p*H 5, and the enzymatic hydrolysis and ethyl acetate extractions were repeated. The first and second neutral extracts when combined weighed 8.5 g. The aqueous residue from the extraction from the second enzymatic hydrolysis was adjusted to *p*H 1 and continuously extracted with ether for 48 hours. The ether layer was washed with dilute bicarbonate until neutral and then evaporated *in vacuo*. The resulting extract was found to contain a small amount of radioactivity, the majority of which was associated with 3α-hydroxypregnane (11β-18) (18–20) dioxide (M1), and the remainder with an unidentified substance. No aldosterone could be found in this extract.

Chromatography of the Neutral Extract from Enzymatic Hydrolysis

The 8.5 g neutral extract was chromatographed in system A on a column of 1 kg of celite. The fractions of the chromatogram were combined into zones I, II, and III as previously described (Kelly *et al.*, 1962). Zones I and II were treated in a manner similar to that already described, and the isolation of a number of new non-polar metabolites of aldosterone will be the subject of a separate report. Zone III (weight 1.5 g) was chromatographed in system B on a 250 g column in order to separate 3α,5β-tetrahydroaldosterone (M12) (6.5 hold-back volumes) from an unidentified radioactive substance (1.5 hold-back volumes) (M6), aldosterone, (2.2 hold-back volumes), 3α,21-dihydroxypregnane (11β-18) (18–20) dioxide (M8) (2.2 hold-back volumes), 3α,5α-tetrahydroaldosterone (M10) (4.5 hold-back volumes), and traces of a blue tetrazolium-positive,

non-radioactive substance presumed to be tetrahydrocortisone (5.0 hold-back volumes).

The principal radioactive peak displayed a varying specific activity among the fractions of the chromatogram and, in addition, was asymmetric in shape. The central portion of this peak was assumed to contain mostly M12. The fractions comprising this portion of the peak were combined (fraction 1), and all the remaining radioactive fractions of this peak were pooled (fraction 2). Fraction 1 (weight 527 mg) was rechromatographed in system B and a small amount of a more polar substance (M11) was present as a shoulder on the main peak. The fractions comprising this shoulder were combined with fraction 2.

Fraction 1. 3α,5β-Tetrahydroaldosterone (M12).—The fractions comprising the main peak from the column (system B) described above were combined, and this material was chromatographed in system O. In this system 3α,5β-tetrahydroaldosterone was separated completely from 18-hydroxytetrahydro A (Ulick *et al.*, in preparation). The fractions comprising each peak were combined. The 3α,5β-tetrahydroaldosterone was rechromatographed first in system P, and then in the B5 system of Bush (1952). An amorphous white residue which resisted repeated attempts at crystallization from a variety of aqueous and non-aqueous solvent systems was obtained from the final chromatogram. The directly determined weight agreed with that determined from the blue tetrazolium reaction, and all subsequent manipulations failed to disclose evidence of the presence of any impurity. A total of 160 mg was isolated and an additional 70 mg had been isolated in a similar manner from the urine of subjects who had received unlabeled aldosterone-21-monacetate orally and tritiated *d*-aldosterone intravenously (Kelly *et al.*, 1962).

Fraction 2. 3β,5β-Tetrahydroaldosterone (M11).—The residue from fraction 2 was chromatographed in system O. Two poorly separated radioactive peaks were found, and each of these was separately chromatographed in system P. The less polar radioactive peak from system O was resolved in system P into three components eluted in 1.0, 2.5, and 4.0 hold-back volumes respectively. The major component was the last. The more polar radioactive peak from system O was also resolved into three components eluted in 1.0, 2.5, and 4.0 hold-back volumes. However the major component was eluted at 2.5 hold-back volumes, and this substance was identified as 3α,5β-tetrahydroaldosterone. The material eluted in 4.0 hold-back volumes from both columns was combined and designated M11. After rechromatography of M11 in the same system, 5 mg of a colorless oil was recovered after evaporation of the solvents. This metabolite was shown to be 3β,5β-tetrahydroaldosterone.

3α,5α-Tetrahydroaldosterone (M10).—M10 was rechromatographed in system B and then in system C. In the latter system it was eluted in 17 hold-back volumes with separation from most of the pigments and from some of the tetrahydrocortisone. Complete separation from tetrahydrocortisone was achieved by two successive chromatograms in system P, in which M10 was eluted in 2.0 hold-back volumes and tetrahydrocortisone in 3.8 hold-back volumes.

The infrared spectrum of M10 indicated the presence of gross impurities which were removed by chromatography in system E followed by chromatography in system B on a column washed with 10 hold-back volumes of mobile phase before the sample was applied. About 3 mg of a semicrystalline solid was eluted from the column in the usual place. The infrared spectrum shown in Figure 6 displayed an absorption band at 1710 cm^{-1} (ketone).

Table II summarizes the chromatographic behavior of the metabolites.

TABLE II

CHROMATOGRAPHIC SEPARATION OF TETRAHYDRO-METABOLITES OF ALDOSTERONE AND OTHER URINARY STEROIDS; NUMBER OF HOLD-BACK VOLUMES REQUIRED FOR ELUTION

Compound	Partition System[a] B	O	P
3α,5α-Tetrahydroaldosterone	4.5	—	2.0
Tetrahydrocortisone	5.0	—	3.8
3α,5β-Tetrahydroaldosterone	6.5	5.2	2.5
3β,5β-Tetrahydroaldosterone	7.5	4.9	4.0
18-Hydroxytetrahydro-11-dehydrocorticosterone	6.5	2.5	—
3β,5α-Tetrahydroaldosterone	—	—	3.6

[a] The compositions of the partition systems are given in Table I.

Enzymatic Preparation of M10

Rat liver microsomes were prepared by the methods previously used by Forchielli *et al.* (1958) and McGuire *et al.* (1960). Four adult female rats were sacrificed and exsanguinated. The livers (wet weight 38.8 g) were homogenized manually in an all-glass homogenizer in the cold in 60 ml of 0.158 M KCl solution. The homogenate was centrifuged in the cold at 10,000 × *g* and the resultant supernatant fluid was centrifuged at 100,000 × *g* for 1 hour at 0°. The sedimented material containing the microsomes was washed with 0.158 M KCl solution, then stored overnight in the frozen state.

The microsomes were resuspended in 40 ml of 0.02 M phosphate buffer at *p*H 7.2 and 21 mg of H^3-*d*-aldosterone-21-monoacetate (specific activity 97 cpm/μg) was added in propylene glycol solution. This mixture was incubated under nitrogen at 38.5° for 3 hours in the presence of a TPNH regenerating system composed of 30 K units of glucose-6-phosphate dehydrogenase (Sigma), 200 mg of glucose-6-phosphate (Sigma), 450 mg of TPN (Sigma), 50 mg of TPNH (Calbio-

chem), and 20 mg of $MgCl_2$. The incubation mixture was poured with stirring into 500 ml of freshly distilled tetrahydrofuran. After the mixture was allowed to stand for an hour with occasional shaking, the suspension of proteins in tetrahydrofuran was removed by filtration. The tetrahydrofuran was removed by vacuum evaporation and the aqueous residue was diluted to 100 ml and extracted three times with equal volumes of ethyl acetate. The combined organic extracts were washed twice with 0.1 volume of water and then evaporated to dryness under vacuum.

The residue, weighing 120 mg, was chromatographed on a 50 g column in system B. The major product, M10, was eluted at 3.3 hold-back volumes with a tail extending into the sixth hold-back volume. A minor product was eluted at 0.8 hold-back volume.

The main product was rechromatographed in system P, whence it was eluted in 2.0 hold-back volumes in a narrow symmetrical band. The tail from the preceding chromatogram was also chromatographed in this system, in which it was separated into two components eluted at 2.0 and 3.6 hold-back volumes respectively. A total of 14.5 mg of M10, a white semicrystalline substance melting at 175–180°, was recovered. This metabolite appeared to have a crystalline structure when examined in ordinary light. However, in polarized light it did not appear to be doubly refractive. The infrared spectrum was identical with that of the material isolated from urine.

Structural Studies

3α,5β-tetrahydroaldosterone, M12.

M12-3 Etiolactone(3α,11β-Dihydroxy-18-oxo-5β-etianic acid (18 → 11) hemiacetal (20 → 18) lactone) from 3α,5β-tetrahydroaldosterone M12.—To 15 mg of tetrahydroaldosterone in 0.5 ml of methanol was added 0.5 ml of a 0.2 N $NaIO_4$ solution. The reaction mixture was allowed to stand at room temperature overnight and then poured into 200 ml of methylene chloride. The methylene chloride was washed three times with 20 ml of water, dried over sodium sulfate, and evaporated under vacuum. The dry residue, after chromatography in system R, yielded white needle-shaped crystals from methanol, melting point 254–255°. A mixture of this compound and the etiolactone from 3α,5β-tetrahydroaldosterone prepared by Ulick *et al.* (in preparation) melted at 254–255°. The infrared spectrum displayed only one carbonyl absorption (1780 cm^{-1}, γ-lactone).

M12-4 Lactone Acetate.—The mother liquor from the crystallization of the etiolacetone was taken to dryness and allowed to stand at room temperature overnight in the presence of acetic anhydride and pyridine. The reaction mixture was diluted by the addition of 5 ml of benzene, and then evaporated to dryness under a stream of nitrogen. The residue was chromatographed on system S. After removal of the solvents from the fractions which contained radioactivity, a crystalline product, m.p. 279–281°, was obtained. Ulick *et al.* (in preparation) report a melting point of 279–281°.

M12-5 3-Keto-5β-etiolactone.—Two mg of etiolactone M12-3 were allowed to stand with 1 ml of CrO_3-pyridine complex (10 mg CrO_3/ml) (Poos *et al.*, 1953) overnight at room temperature. The reaction mixture was poured into about 100 ml of water, which was extracted three times with ether. The ether extracts were combined and washed three times with water to remove the pyridine, then dried over sodium sulfate and evaporated to dryness under vacuum. The dry residue yielded crystals melting at 249–251° (Ulick *et al.* (in preparation) m.p. 244–245° from methanol-ether). The infrared spectrum displayed bands at 1775 cm^{-1} (γ-lactone) and 1705 cm^{-1} (ketone).

M12-1 Tetrahydroaldosterone Triacetate.—Fifty-three mg of tetrahydroaldosterone was dissolved in 0.4 ml of pyridine and 0.2 ml of C^{14} acetic anhydride. After standing overnight the reaction mixture was diluted with 5 ml of benzene and then evaporated to dryness under a stream of nitrogen.

After chromatography of the residue on system S a white amorphous solid was obtained. Its infrared spectrum indicated the absence of hydroxyl groups and the presence of a 21-acetoxy-20-ketone grouping (1755 cm^{-1}, 1735 cm^{-1}). The acetoxy C-O absorption band in the 1200–1250 cm^{-1} region displayed a shoulder at about 1230 cm^{-1} (Fig. 3). Determination of the number of acetoxy groups indicated that this derivative was a triacetate, as is shown in Table III.

M12-6 Tetrahydroaldosterone Diacetate from M12-1 Tetrahydroaldosterone Triacetate.—Eleven

TABLE III

DETERMINATION OF THE NUMBER OF ACETYLATABLE HYDROXYL FUNCTIONS IN METABOLITES OF ALDOSTERONE AND THEIR DERIVATIVES[a]

Compound	H^3/C^{14}	C^{14} Cpm μeq Ac_2O	H^3 Cpm μmole compound	Number of Acetylatable Hydroxyl Groups
M10-1[b]	4.55	2300	30,300	2.9
M10-2[b]	6.5	2300	30,300	2.0
M11-1	6.25	2300	44,000	3.1
M11-2	8.5	2300	44,000	2.25
M12-1	6.0	2300	44,000	3.2
M12-2	9.5	2300	44,000	2.0
M12-6	9.8	2300	44,000	1.9
M12-7[c]	3.0	1060	6,800	2.1
M12-8[c]	3.0	1060	6,800	2.1
M12-12	17.7	2300	44,000	1.1

[a] Counting conditions and calculations as described previously (Kelly *et al.*, 1962). All counting data are corrected for fluctuations in counting efficiency. [b] M10 isolated from the rat liver incubation. [c] M12 isolated from urine pools I, II, and III as previously described (Kelly *et al.*, 1962).

mg of tetrahydroaldosterone - H^3 - triacetate - C^{14} was allowed to stand in solution of 0.8 ml ethanol, 0.8 ml glacial acetic acid, and 0.4 ml of distilled water for 20 hours. The solvents were removed by azeotropic distillation with benzene, and the residue was chromatographed in system S. Nine mg of a white amorphous solid was eluted in 7.8 hold-back volumes. The infrared spectrum (Fig. 3) indicated the presence of hydroxyl (3500 cm^{-1}), 21-acetoxy-20-ketone (1755 cm^{-1}) and acetate (1735, 1240 cm^{-1}) groups. In addition the fingerprint region of the spectrum differed from that of tetrahydroaldosterone triacetate. The simple contour of the band near 1240 cm^{-1} indicated an equatorial 3-acetoxy group (Jones and Herling, 1956). Comparison of the H^3/C^{14} ratios of the triacetate and of the product indicated the loss of one acetoxy group. Determination of the number of acetoxy groups revealed the presence of two such groups (*cf.* Table III).

M12-6 from Tetrahydroaldosterone, M12.—Three mg of tetrahydroaldosterone was allowed to stand at room temperature overnight in a solution of 24 ml of pyridine and 16 ml of 10% acetic anhydride in benzene. The solvents were removed *in vacuo* and the dry residue was chromatographed on system S. Two components were resolved. The triacetate, M12-1, was eluted in 4.4 hold-back volumes. The second component was eluted in 7.8 hold-back volumes and was identified as the diacetate M12-6. About three times as much triacetate as diacetate was formed under these conditions.

M12-2 (18 → 11) Lactone from M12-6, Tetrahydroaldosterone Diacetate.—Four and one-half mg of tetrahydroaldosterone H^3-diacetate-C^{14} was oxidized with 0.1 ml of 2% chromic acid in 90% acetic acid. The reaction mixture was poured into water and the water was extracted three times with ether. The combined ether extracts were washed with dilute $NaHCO_3$ solution, then with water until neutral. The ether was dried over sodium sulfate, then removed *in vacuo*. The dry residue was chromatographed in the E-4 system (Eberlein and Bongiovanni, 1955) and a crystalline product, m.p. 162–164°, was eluted in 6.5 hold-back volumes. The infrared spectrum indicated the absence of hydroxyl groups and the presence of a γ-lactone (1780 cm^{-1}), a 21-acetoxy-20-ketone (1755 cm^{-1}), and acetoxy groups (1735, 1240 cm^{-1}). The simple contour of the band near 1240 cm^{-1} indicated the presence of an equatorial 3-acetoxy group (Jones and Herling, 1956). Determination of the specific activity with respect to C^{14} revealed the presence of two acetoxy groups, as shown in Table III.

M12-2 from M12-1, Tetrahydroaldosterone Triacetate.—Five mg of tetrahydroaldosterone triacetate was oxidized with 0.1 ml 2% chromic acid in 90% acetic acid in the same manner as the diacetate, M12-6. The product melted at 162–164° and its infrared spectrum was identical with that of 3α,21-diacetoxy-11β-hydroxypregnan-18-oic (18 → 11) lactone prepared from tetrahydroaldosterone diacetate.

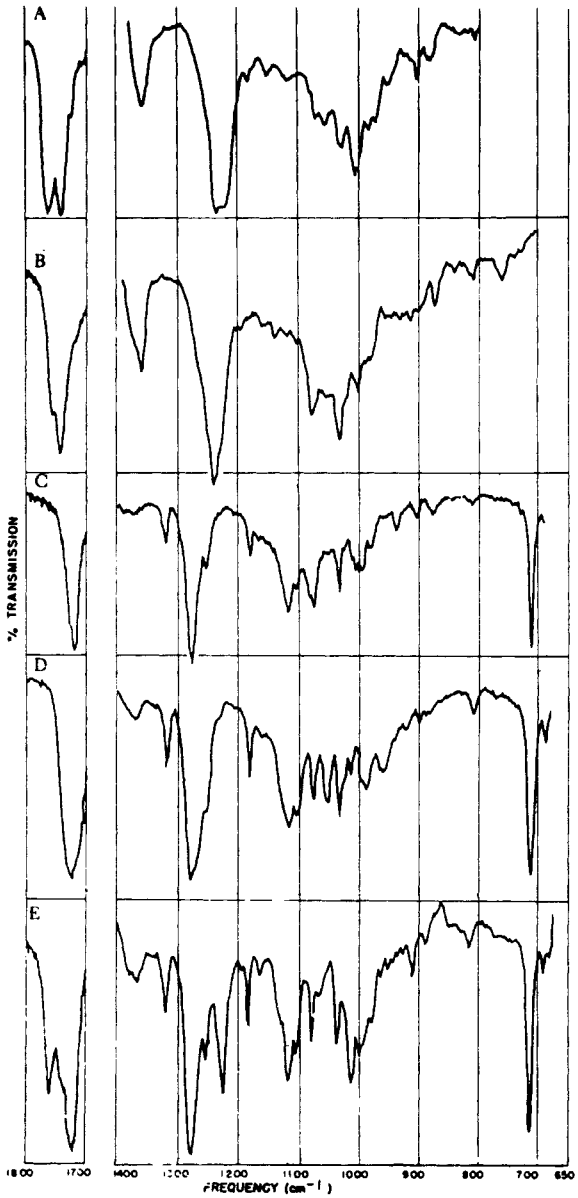

FIG. 3.—Infrared spectra of esters of 3α,5β-tetrahydroaldosterone in CS_2 solution. A, M12-1—triacetate; B, M12-6—diacetate; C, M12-10—dibenzoate; D, M12-9—tribenzoate; E, M12-12—dibenzoate monoacetate.

Methylacetals M12-7 and M12-8.—A solution of 14 mg of 3α,5β-tetrahydroaldosterone M12 and a few crystals of *p*-toluenesulfonic acid in 50 ml methanol and 100 ml of benzene was refluxed under nitrogen for one hour. The cooled reaction mixture was diluted with 900 ml of benzene and washed, first with 2% aqueous $NaHCO_3$ and then with water until the washings were neutral. The organic phase was dried over sodium sulfate and the solvents were removed by vacuum evapo-

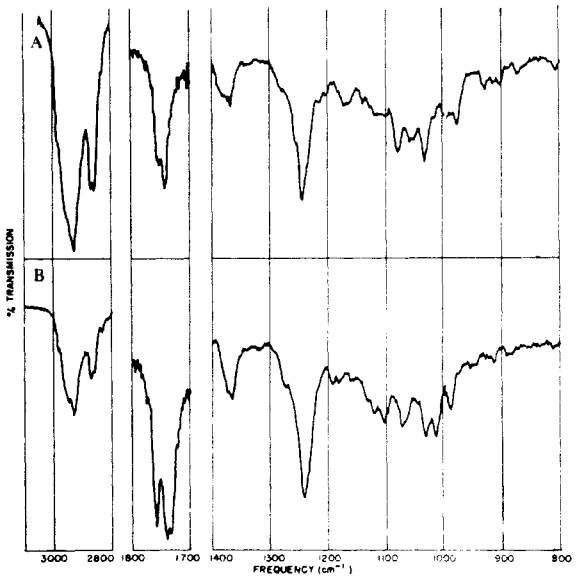

FIG. 4.—Infrared spectra of the methylacetal diacetates in CS_2 solution. A, M12-7; B, M12-8.

ration. The crude residue, weighing 15 mg, was chromatographed in system U. About 1 mg of etiolactone, M12-3, was eluted in the second hold-back volume, but the main product was not eluted in 11 hold-back volumes. Therefore the product was removed from the column with methanol and rechromatographed in system V. A colorless oil weighing 7 mg was eluted as a single peak in 5.1 hold-back volumes. This material was acetylated with C^{14}-labeled acetic anhydride in pyridine at room temperature for 18 hours. The reagents were removed by evaporation with 5 ml of benzene and the dry residue was chromatographed in system Q. About 1.5 mg of a colorless oil, M12-7, was eluted in 1.0 hold-back volume and about 5 mg of a crystalline substance, M12-8, m.p. 174–176°, was eluted in 3.3 hold-back volumes.

The infrared spectrum of M12-7 (Fig. 4) displayed absorption bands at 2835 cm^{-1} ($O-CH_3$, Henbest *et al.*, 1957), 1755, 1743 cm^{-1} (21-acetoxy-20-ketone), and 1245 cm^{-1} (acetoxy). The blue tetrazolium reaction was negative, even when the concentration of alkali was increased to 1.0 N. Determination of the number of acetoxy groups (Table III) revealed that M12-7 was a diacetate.

Oxidation of M12-7 to Lactone M12-2.—One mg of M12-7 was treated with 0.1 ml of 2% CrO_3 in 90% acetic acid at room temperature for 18 hours. The product was isolated as described for M12-2 and its infrared spectrum was identical to that of M12-2. Crystalline M12-2 was obtained by high vacuum microsublimation.

M12-8.—The infrared spectrum of M12-8 (Fig. 4) displayed bands at 2815 cm^{-1} (methoxyl), 1760, 1740, 1728 cm^{-1} (21-acetoxy-20-ketone, acetate), and 1240 cm^{-1} (acetate). The blue color in the blue tetrazolium reaction developed slowly, and increased alkali concentration accelerated the rate of color development. This compound was also a diacetate (Table III).

Oxidation of M12-8 to Lactone M12-2.—One mg of M12-8 was treated with 0.1 ml of 2% CrO_3 in 90% acetic acid as described under M12-7. The product, a crystalline lactone obtained by high vacuum microsublimation, was identified as M12-2 by its infrared spectrum.

M12-9 Tribenzoate.—Fourteen mg of tetrahydroaldosterone M12 was dissolved in 0.6 ml of dry pyridine and 0.18 ml of freshly distilled benzoyl chloride. The reaction mixture was sealed in an ampul and allowed to stand 24 hours at 4°, then for 48 hours at room temperature. The ampul was opened and 0.6 ml of methanol was added. Two hours later the reaction mixture was taken up in 150 ml of ethyl acetate, which was then washed first with 2% (v/v) sulfuric acid, then with 5% (w/v) sodium carbonate solution, and finally with water until the washings were neutral. The ethyl acetate solution was dried over sodium sulfate and then taken to dryness *in vacuo*. The residue was chromatographed on a 50-g column in system T. A single radioactive peak was eluted in 2.0 hold-back volumes. Removal of the solvent from the radioactive fractions left a white amorphous residue. Comparison of the molar extinction coefficient of this benzoate of tetrahydroaldosterone (35,600 at 235 mμ in methylene chloride) with that of dehydroisoandrosterone benzoate (12,000) indicated that it was a tribenzoate. Its infrared spectrum (Fig. 3) contained no bands near 3500 cm^{-1} (hydroxyl) but did possess bands at 3095, 3070, 3040 cm^{-1} (aromatic C-H), 1725 cm^{-1} (carbonyl), 1605, 1585, and 710 cm^{-1} (aromatic ring), and 1280, 1110 cm^{-1} (benzoate).

M12-10 Dibenzoate.—Nine mg of tribenzoate M12-9 was dissolved in a drop of benzene and 1.5 ml of 90% acetic acid was added. After standing for 4 days at room temperature, the reaction mixture was taken to dryness by azeotropic distillation with benzene and ethanol in vacuum. The residue was chromatographed in system T from which it was eluted in 3.6 hold-back volumes. After evaporation of the solvent, an amorphous residue weighing 6 mg was obtained. The infrared spectrum (Fig. 3) displayed a broad weak band near 3500 cm^{-1} (hydrogen-bonded OH), and major bands at 3090, 3070, 3040 cm^{-1} (aromatic C-H), 1725 cm^{-1} (carbonyl), 1605, 710 cm^{-1} (aromatic ring), and 1275, 1115 cm^{-1} (benzoate ester). The extinction coefficient of M12-10 was 22,200 at 235 mμ in methylene chloride. Thus M12-10 was a dibenzoate.

M12-11 Lactonedibenzoate, from M12-9.—Four mg of tribenzoate M12-9 was oxidized with 0.2 ml of 2% CrO_3 in 90% acetic acid at room temperature for 4 days. The reaction mixture was poured into 100 ml of water, which was extracted three times with 100-ml portions of ether. The

combined ether extracts were washed first with dilute $NaHCO_3$ solution and then with water until the washings were neutral. The ether was dried over Na_2SO_4 and removed by vacuum evaporation. The residue was chromatographed in the E-4 system. Upon rechromatography in system T two non-crystalline substances were eluted in 5.4 and 7.5 hold-back volumes respectively. The ratio of products was about 1:3 in favor of the polar component. The infrared spectrum of the latter (Fig. 5) indicated the presence of γ-lactone (1780 cm^{-1}) and a benzoate ester (1720, 1275, 1110, 705 cm^{-1}). The molar extinction coefficient (26,700 at 235 mμ in methylene chloride) indicated the presence of two benzoxyl groups.

M12-11 from M12-10.—About 3 mg of dibenzoate M12-10 was oxidized with 2% CrO_3 in 90% acetic acid at room temperature for 18 hours. The products were isolated as described above and chromatographed on system T. One product was eluted at 5.4 hold-back volumes and another at 7.5 hold-back volumes. The ratio of products was 1:20 in favor of the more polar component, whose infrared spectrum was identical with that of M12-11 from M12-9.

Minor Product from Preparation of M12-11.—The product eluted at 5.4 hold-back volumes from the system T chromatogram was, unlike M12-11, blue tetrazolium-negative. The infrared spectrum displayed bands at 1780 cm^{-1} (lactone), 1760 cm^{-1} (unassigned), and 1720 cm^{-1} (benzoate ester). In addition, the fingerprint region of the spectrum differed from that of M12-11. At present a structure cannot be formulated for this compound.

M12-12 Dibenzoate Monoacetate.—Three mg of dibenzoate M12-10 was acetylated with 0.05 ml C^{14}-acetic anhydride and 0.10 ml pyridine. The reagents were removed by azeotropic evaporation with benzene under a stream of nitrogen and the dry residue was chromatographed in system T, from which it was eluted in 2.6 hold-back volumes. Evaporation of the chromatography solvents yielded a white, amorphous material whose infrared spectrum (Fig. 3) indicated the presence of an acetoxy group (1225 cm^{-1}) together with a benzoxy group (1725, 1275, 1110, 705 cm^{-1}). In addition, a strong band was observed at 1760 cm^{-1} [(O-C_{18}-O-Ac) (Heusler and Wettstein, 1962)]. Determination of the extinction coefficient (23,400 at 235 mμ in methylene chloride) and of the number of acetoxy groups (Table III) indicated that M12-12 was a monacetoxy dibenzoxy derivative.

3β,5β-Tetrahydroaldosterone M11

M11-1 3β,5β-Tetrahydroaldosterone Triacetate.—One mg of 3β,5β-tetrahydroaldosterone was acetylated by standing overnight in C^{14}-acetic anhydride–pyridine mixture. The reaction mixture was diluted with 5 ml of benzene, and the solvents were evaporated to dryness under a stream of nitrogen. The residue was chromatographed on system Q. Determination of the number of acetoxy groups indicated the formation of a triacetate as shown in Table III. The infrared spectrum of the non-crystalline material eluted from the chromatogram differed from the spectrum of tetrahydroaldosterone triacetate, M12-1. The most striking difference was in the 1200 and 1250 cm^{-1} region, where this compound displayed a doublet which suggested the presence of an axial 3-acetoxy group (Jones and Herling, 1956). This spectrum was identical to that of 3β,5β-tetrahydroaldosterone triacetate prepared enzymatically by Ulick *et al.* (in preparation) from aldosterone.

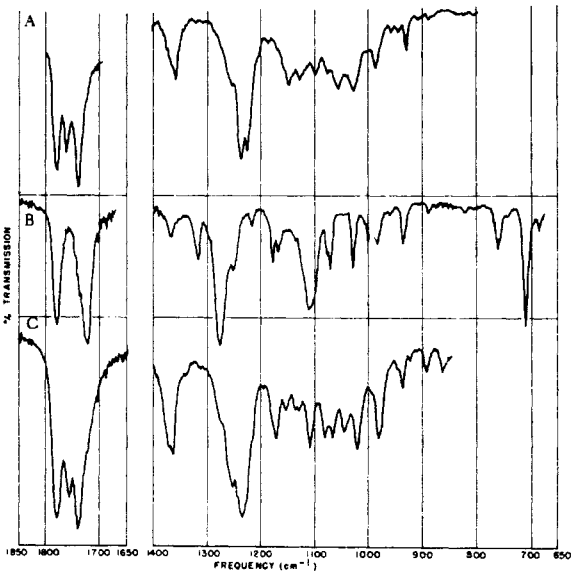

FIG. 5.—Infrared spectra of 18→11 lactones in CS_2 solution. A, M11-2—lactone diacetate; B, M12-11—lactone dibenzoate; C, M10-2—lactone diacetate.

M11-2 3β,5β-Tetrahydroaldosterone Diacetate (18 → 11) Lactone.—Eight hundred micrograms of 3β,5β-tetrahydroaldosterone triacetate, M11-1, was oxidized at room temperature over-night in 0.1 ml of a solution of 2% chromic acid in 90% acetic acid. The reaction mixture was poured into water and the product was extracted with ether. The ether was washed, first with dilute $NaHCO_3$ solution, then with water until the washings were neutral. The ether was dried over Na_2SO_4 then evaporated *in vacuo*. The residue was chromatographed in the E-4 system (Eberlein and Bongiovanni, 1955) and the product was eluted in the third hold-back volume as a colorless oil. Its infrared spectrum (Fig. 5) indicated the absence of hydroxyl groups and the presence of a γ-lactone (1780 cm^{-1}), 21-acetoxy-20-ketone (1755 cm^{-1}), and acetate (1735, 1240, 1225 cm^{-1}) groups. The doublet in the 1200 to 1250 cm^{-1} region was consistent with an axial 3-acetoxy group (Jones and Herling, 1956).

3α,5α-Tetrahydroaldosterone, M10

M10-1, Triacetate from M10.—A solution of 3 mg of M10 in 0.1 ml C^{14}-acetic anhydride and 0.2 ml pyridine was allowed to stand overnight at room temperature. About 5 ml of benzene was added and the solvents were removed by evaporation under nitrogen. The residue was chromatographed on the Q system. A colorless oil was obtained after evaporation of the solvents from the tubes which contained the tritium label. The infrared spectrum (Fig. 6) displayed bands 1760, 1730 cm^{-1} (21-acetoxy-20-ketone) and a band at 1230 cm^{-1} with shoulders at 1240 cm^{-1} and 1225 cm^{-1} (acetoxy). Determination of the number of acetoxy groups (Table III) indicated that M10-1 was a triacetate.

M10-2, Lactone Diacetate from M10-1.—Two mg of M10-1 was oxidized with 0.1 ml of 2% chromic acid in 90% acetic acid overnight at room temperature. The reaction mixture was poured into 100 ml of water, which was extracted three times with ether. The combined ether extracts were washed first with a dilute $NaHCO_3$ solution, and then with water until the washings were neutral. The ether was dried over sodium sulfate and evaporated to dryness. The residue was chromatographed in the E-4 system. A colorless oil was obtained upon evaporation of the solvents from the fractions containing radioactivity. Its infrared spectrum (Fig. 5) displayed bands at 1780 cm^{-1} (γ-lactone), 1755, 1740 cm^{-1} (21-acetoxy-20-ketone), and 1250, 1235 cm^{-1} (acetoxy). The shape of the acetoxy band suggested the axial conformation for the 3-acetoxy group (Jones and Herling, 1956). Determination of the number of acetoxy groups (Table III) indicated that M10-2 was a diacetate.

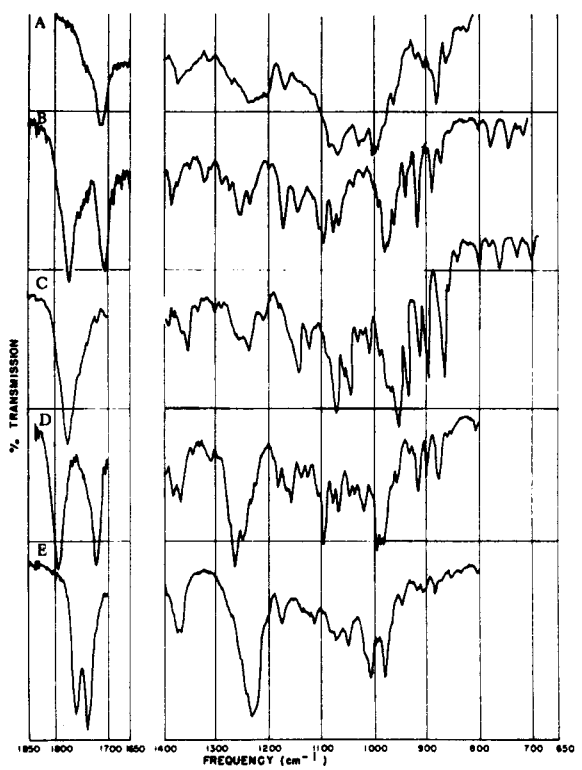

FIG. 6.—Infrared spectra of 3α,5α-tetrahydroaldosterone and some of its derivatives. A, M10 in $CHCl_3$ solution; B, M10-5—3-ketoetiolactone in KBr; C, M10-3—etiolactone in KBr; D, M10-4—etiolactone acetate in KBr; E, M10-1—triacetate in CS_2 solution. Spectra in KBr were determined with a sample of about 20 μg in about 2 mg of KBr. The pellet was 1.5 mm in diameter, and a sample beam condensing unit was used.

M10-3, Etiolactone (3α,11β-Dihydroxy-18-oxo-5α-etianic acid (18 → 11) hemiacetal (20 → 18) lactone) from M10.—Three mg of M10 was dissolved in 1 ml of methanol, and 0.5 ml of an aqueous solution 0.1 N in HIO_4 and 0.12 N in pyridine was added, and the reaction mixture was allowed to stand overnight at room temperature. The reaction mixture was poured into water and the product was extracted three times with methylene chloride. The combined organic extracts were washed with water, dried over sodium sulfate, and evaporated to dryness. The crystalline residue was recrystallized from acetone-hexane and on heating sublimed at between 230 and 255°. The infrared spectrum (Fig. 6) indicated the presence of a γ-lactone (1775 cm^{-1}).

M10-4, 3α-Acetoxy-5α-etiolactone.—A solution of 1 mg of M10 in 0.1 ml of pyridine and 0.05 ml of acetic anhydride was allowed to stand overnight at room temperature. Five ml of benzene was added, and the solution was evaporated to dryness under a stream of nitrogen. The residue crystallized from hexane–methylene chloride. The product melted at 212–214°. Its infrared spectrum in CS_2 solution (Fig. 6, in KBr) displayed bands at 1790 cm^{-1} (lactone), 1740 cm^{-1} (ester), and 1255, 1240 cm^{-1} (axial 3-acetoxy). This spectrum was different from that of the 3β-acetoxy-5β-etiolactone prepared by Ulick *et al.* (in preparation).

M10-5, 3-Keto-5α-etiolactone.—A solution of 1.5 mg of M10-3 in 0.1 ml of pyridine-CrO_3 complex (10 mg CrO_3/ml pyridine) was allowed to stand overnight at room temperature. The reaction mixture was poured into 100 ml of water, which was extracted three times with methylene chloride. The combined methylene chloride extracts were washed four times with water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The residue crystallized as needles from ethanol-water. After preliminary softening and partial sublimation, the bulk of the sample melted at 305–307°. Ulick *et al.* (in preparation) report a melting point of 295–301° from methylene chloride-methanol, and Pechet *et al.* (1960) report 288–295° from moist ethanol. The infrared spectrum (Fig. 6) displayed bands at 1770 cm^{-1} (γ-lactone) and 1705 cm^{-1} (ketone). The fingerprint region of this spectrum differed from that of the 3-keto-5β-etiolactone M12-5.

M10-5 from 3β,5α-Etiolactone.—A pyridine solution of 0.5 mg of 3β-hydroxy-5α-etiolactone (generously provided by Dr. M. M. Pechet), melting point 240–245° (Ulick *et al.*, [in preparation] report a melting point of 241–243° for this substance) was treated with the pyridine-CrO_3 complex as described above. Comparison of the infrared spectrum of the product and of M10-5 indicated that the two were identical.

Minor Products from the Incubation of Rat Liver Microsomes

The less polar of the two minor products from the rat liver incubation (system B, 0.8 hold-back volumes) was recrystallized from aqueous acetone. It melted at 165–170°. A solution of 1 mg of this product in methanol was oxidized with HIO_4 in aqueous pyridine as described under M10-3. The infrared spectrum of the oxidation product was identical with that of the 3-keto-5α-etiolactone M10-5. Therefore, the less polar product must be 5α-dihydroaldosterone (11β,21-dihydroxy-18-oxoallopregnane-3,20-dione). Pechet *et al.* (1960) reported a melting point of 154–156° for this substance.

Only about 500 μg of the more polar minor product (system P, 3.6 hold-back volumes) was isolated. The infrared spectrum was consistent with that reported for 3β,5α-tetrahydroaldosterone (Pechet *et al.*, 1960). The identity of the product was confirmed through its conversion to the known 3β,5α-etiolactone by oxidation with periodic acid.

Results and Discussion

3α,5β-Tetrahydroaldosterone, M12

Reports concerning the existence of 18-hydroxy-20-ketosteroids as cyclic hemiketals which are unreactive toward acetic anhydride in pyridine stimulated us to re-evaluate the tentative structure assigned (Ulick and Lieberman, 1957) to the major urinary metabolite of aldosterone, because its rapid rate of reaction with blue tetrazolium and its formation of a triacetate seemed inconsistent with the proposed 11-keto-18-hydroxy structure. On the other hand, this structure was consistent with the formation (Ulick and Lieberman, 1957) of a keto-lactone of an etio-acid upon periodic acid oxidation. Recently, however, Ulick *et al.* (1961) have shown that this lactone was in fact derived from 18-hydroxytetrahydro A, which was present as a contaminant in the original tetrahydroaldosterone sample. They further showed that periodic acid oxidation of purified tetrahydroaldosterone yielded an etiolactone which did not have a ketone function. On the basis of this new evidence, Ulick *et al.* (1961 and in preparation) have proposed the structure M12 shown in Figure 2 for the major urinary metabolite of aldosterone.

In this study, confirmatory evidence for this structure was obtained by the preparation of derivatives M12-1 and M12-3 through M12-6 (Fig. 2) which had been previously prepared by Ulick and his co-workers (1957, 1961, in preparation). Further evidence in support of structure of M12 came from the preparation of the γ-lactone diacetate, M12-2, by chromic acid oxidation of the diacetate M12-6. This transformation also served to characterize M12-6 as the 3α,21-diacetate. The reaction sequence employed in this study to characterize M12 through these six derivatives is analogous to that employed in the proof of structure of aldosterone (Simpson *et al.*, 1954).

The oxidative behavior of triacetate M12-1 and tribenzoate M12-9 to yield the 3α,21-diacyl (18 → 11) lactones, M12-2 and M12-11, respectively, is similar to that of aldosterone diacetate (Kliman and Peterson, 1960). These authors correctly inferred that the product was the 18 → 11 lactone even though it could not be characterized because of the limited quantities of aldosterone then available. This product has been recently established (Kelly *et al.*, unpublished observations) as 3,20-diketo-11β-hydroxy-21-acetoxy-Δ^4-pregnen-18-oic (18 → 11β) lactone by comparison of the oxidation product of aldosterone diacetate with the known lactone acetate which was prepared from aldosterone-21-monoacetate by the method of Simpson *et al.* (1954).

Thus the assignment of structure M12-2 to the lactone diacetate is based upon analogy with known reactions and upon evidence from the infrared spectrum for both the presence of a γ-lactone and a 21-acetoxy-20-ketone grouping and also for the absence of a hydroxyl function. Determination of the number of acetoxy groups confirmed that M12-2 was a diacetate. This compound is the first described crystalline derivative of 3α,5β-tetrahydroaldosterone to possess all 21 carbon atoms. Similarly, this analogy can be extended to permit the assignment of structure M12-11 to the lactone dibenzoate. Further evidence for this structure came from the determination of the number of benzoxyl groups and from the positive blue tetrazolium reaction which indicated the presence of the C-21-benzoxy-20-ketone group in M12-11. The characterization of M12-11 as an 18 → 11β lactone establishes the structure of the 3α,21-dibenzoate, M12-10.

The 11β and 18 oxygen functions in 3α,5β-tetrahydroaldosterone exist mainly as a hemiacetal. This is suggested by the formation of di- and triacyl derivatives by oxidation to an (18 → 11β) lactone instead of the 11-keto-18-oic acid, and by formation of an etiolactone on oxidation with periodic acid. Such a hemiacetal group would be expected to form a methyl acetal when treated with methanol in the presence of acid. When 3α,5β-tetrahydroaldosterone was refluxed with methanol containing *p*-toluenesulfonic acid, the major product isolated was less polar than M12 and upon acetylation gave rise to two

diacetates. One of these, M12-8, was crystalline and reacted slowly with the blue tetrazolium reagent. The second diacetate, M12-7, was less polar than M12-8 and did not crystallize; nor did it react with the blue tretrazolium reagent. Both compounds absorbed infrared radiation near 2835 cm^{-1}, a frequency assigned to the methoxyl group (Henbest *et al.*, 1957). Both spectra also possessed absorption bands characteristic of the 21-acetoxy-20-ketone group, but neither gave evidence of a hydroxyl function. Oxidation with chromic acid converted both acetals to the previously described lactone diacetate, M12-2.

The view that these methyl acetals are probably isomeric at C_{18} is supported by the recent observations of Bojesen and Degn (1961), who reported the preparation of two diastereoisomeric monomethylacetals from aldosterone-21-monopipsylate. Examination of molecular models (Dreiding) of the acetals reveals steric hindrance of rotation in the C_{21} acetoxy-20-ketone grouping by the methoxyl function at C_{18}. Moreover, the degree of hindrance is clearly greater in the 18-S than in the 18-R isomer. If the threefold difference in yield of the acetals from M12 and their anomalous behavior toward the blue tetrazolium reagent can be ascribed to this steric hindrance, the 18-R configuration can be tentatively assigned to the blue tetrazolium–positive acetal, M12-8. The blue tetrazolium–negative acetal produced in lesser yield, M12-7, would then be assigned the more hindered 18-S configuration.

3α,5α- and 3β,5β-Tetrahydroaldosterone, M10 and M11

Two blue tetrazolium–positive polar metabolites of aldosterone, M10 and M11, both of which formed triacetates, were isolated in small amounts along with 3α,5β-tetrahydroaldosterone. These chemical properties suggest that the two compounds are isomers of the major metabolite, M12.

The triacetate M11-1 was identical to the triacetate of 3β,5β-tetrahydroaldosterone prepared enzymatically from aldosterone by Ulick *et al.* (in preparation). By analogy with the oxidation of M12-1 to M12-2, the preparation of the (18 → 11) lactone diacetate, M11-2, served to characterize further M11 as a cyclic hemiacetal. About 5 mg of M11 was isolated from urine, and, allowing for losses in the isolation procedure, this amount represents a conversion of about 1% from administered precursor.

Since both 5β isomers of tetrahydroaldosterone were already known, M10 appeared to be one of the two possible 5α isomers. Only about 3 mg of M10 was isolated from urine, and, therefore, a biosynthesis of a larger amount was undertaken to facilitate the study of its structure. Forchielli *et al.* (1958) and McGuire *et al.* (1960) have demonstrated that female rat liver microsomes reduce Δ^4-3 ketosteroids to products of the 5α series. In our hands, this preparation reduced aldosterone to M10 in about 80% yield and to two other by-products: *viz.* 5α-dihydroaldosterone and 3β,5α-tetrahydroaldosterone. This method of preparation is supporting evidence for 5α configuration of M10. Oxidation of M10 with periodic acid yielded a γ-lactone, M10-3, which upon acetylation gave rise to an acetoxy derivative M10-4 whose infrared spectrum indicated the axial conformation for the acetoxy group. Oxidation of M10-3 to the 3-ketolactone M10-5, a known (Pechet *et al.*, 1960) compound of the 5α series, confirmed the 5α configuration in M10. The configuration of the 3-hydroxyl group was determined to be α from the presence of multiple bands near 1250 cm^{-1} in the infrared spectra of acetoxy derivatives M10-4, M10-1, and M10-2. Furthermore, comparison of lactone M10-3 with the 3β-hydroxy-5α-etiolactone demonstrated that the two lactones had different infrared spectra and different melting behavior. Thus M10-3 must be the 3α hydroxy isomer.

The presence of the cyclic hemiacetal structure in M10 was demonstrated by oxidation of triacetate M10-1 to lactone diacetate M10-2. The preparation of three different lactone diacetates, M10-2, M11-2, and M12-2, rules out the possibility that any pair of the trio of metabolites M10, M11, and M12, could be stereoisomeric at C_{18} only.

The conversion of administered aldosterone to M10 was of the order of 0.5 to 1.0%, allowing for losses occurring during the isolation procedure. Thus unlike cortisol and corticosterone, aldosterone was not significantly reduced to the 3α,5α-tetrahydro derivative. Moreover, no 11-keto metabolite of aldosterone has yet been found, nor has 3β,5α-tetrahydroaldosterone been isolated from human urine in this study.

The infrared spectra of 3α,5β-tetrahydroaldosterone triacetate and of aldosterone diacetate each have a strong band near 1235 cm^{-1} and a shoulder near 1220 cm^{-1} (Fig. 3). This shoulder is not present in the spectrum of aldosterone-21-monoacetate nor in that of the (18 → 11) lactone-21-monoacetate, nor is it found in the spectra of those derivatives of 3α-5β-tetrahydroaldosterone lacking an 18-acetoxy function. Thus it appears that the 1220 cm^{-1} band can be associated with the 18-acetoxy group, whereas the 3 and 21 acetoxy groups absorb near 1235 cm^{-1}. Further evidence in support of this correlation was obtained from the 3,21-dibenzoate-18-acetate, M12-12. This compound displayed strong bands at 1275 cm^{-1} and at 1220 cm^{-1} (Fig. 3). Since the only acetoxy group in this compound is at C_{18}, and since the benzoxy group absorbs at 1275 cm^{-1}, the 1220 cm^{-1} band must be associated with the 18-acetoxy group.

The two 3-axial acetoxy derivatives, M10-1 and M11-1, have multiple bands in this region. In the case of M10-1, a shoulder is present at 1220 cm^{-1}, whereas in derivatives of M11, a strong band is present at this frequency. In the latter instance, the 1220 cm^{-1} band must be

attributed to the 3 and/or 21 acetoxy groups since it is present in the spectrum of the lactone diacetate M11-2.

If the 18-acetoxy group absorbs near 1220 cm^{-1} in the 3-axial acetoxy compounds, this absorption seems to be obscured by that of the other acetoxy groups. On the other hand, in those 18 acetoxy compounds which do not possess a 3-axial acetoxy group, a band near 1220 cm^{-1} appears to be associated with the 18-acetoxy function.

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Addendum

Since this manuscript was submitted for publication, Dr. Robert H. Hesse of the Research Institute for Medicine and Chemistry, Boston (personal communication, 1962) has compared M10 with 3α,11β,21-trihydroxy-18-oxoallopregnan-20-one prepared by incubation of aldosterone-21-monoacetate with a homogenate of rat liver (Hesse *et al.*, 1961) and found that the two compounds were identical, as were their etiolactone acetate derivatives.

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