Isolation and Characterization of Aldosterone Metabolites from Human Urine; Two Metabolites Bearing a Bicyclic Acetal Structure*

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After the administration of several hundred mg of tritium-labeled aldosterone to human subjects, metabolites of the hormone were isolated from the urine of these subjects by enzymatic hydrolysis of the steroid conjugates, followed by extraction of the steroids with ethyl acetate and partition chromatography of the neutral extract. Two of these metabolites were identified as aldosterone and tetrahydroaldosterone. Although six of the radioactive metabolites could not be identified because the amounts isolated were insufficient, the structures of two metabolites were established on the basis of chemical and spectroscopic evidence. Both of these metabolites were found to possess a bicyclic acetal structure, and one of them was shown to be a 21-deoxy compound. The four known metabolites were shown to account for at least 40% of the administered radioactivity.

Since the isolation and chemical characterization (Simpson *et al.*, 1954) of the salt-regulating hormone, aldosterone, the elucidation of the pathways by which it is metabolized and excreted has been impeded by the fact that only a small amount of aldosterone was available from either biological sources or chemical synthesis. Radioactive tracer studies have shown that about 90% of the radioactivity injected as aldosterone is recovered in the urine after 48 hours. Very small amounts of the injected aldosterone itself have been isolated by direct extraction of human urine, but mild acid hydrolysis releases a substantial increment, totalling about 5 to 10% of the dose administered. Hydrolysis of the conjugates in urine with β-glucuronidase releases about 55% of the injected dose in organic soluble form (Flood *et al.*, 1961). Hence it appears that most of the metabolites of aldosterone are in the glucuronoside fraction. An acetone powder prepared from rat liver has been shown (Ulick *et al.*, 1961) to be capable of metabolizing aldosterone to 3α,21-dihydroxy-18-oxo-pregnane-11,20-dione (tetrahydroaldosterone). This compound is identical with the urinary metabolite previously isolated (Ulick and Lieberman, 1957) and accounts for at least 15% of the hormone secreted in man. Originally this metabolite was thought to be 3α,18,21 - trihydroxy - pregnane - 11,20 - dione (18 - hydroxytetrahydro A), but more recently it has been shown (Ulick *et al.*, 1961) that the material isolated was, in fact, a mixture of tetrahydroaldosterone and 18-hydroxytetrahydro A, a metabolite of 18-hydroxycorticosterone.

At least 60% of the aldosterone secreted daily remains to be accounted for as discrete metabolites. In an attempt to isolate and identify these metabolites, several hundred mg of synthetic *d*-aldosterone, which has recently become available (Heusler *et al.*, 1960; Barton and Beaton, 1960), were administered to human subjects, together with trace doses of tritiated aldosterone. The use of *d*-aldosterone rather than *dl*-aldosterone is preferable in an experiment of this type, since it has recently been shown that the natural *d*-isomer is metabolized differently from its antipode (Ulick, 1961). Heretofore, the amounts of metabolites which could be isolated from urine were limited by the secretion rate of aldosterone, which is of the order of 100–250 μg per 24 hours in normal individuals and only rarely exceeds 3000 μg per 24 hours in pathologic conditions (Ulick *et al.*, 1958; Ayers *et al.*, 1957; Peterson, 1959; Laragh *et al.*, 1960). This report describes the isolation from human urine of a number of new metabolites of aldosterone. Two of these, which were recovered in crystalline form, were found to be bicyclic acetals. Their structures are shown in Figure 1.

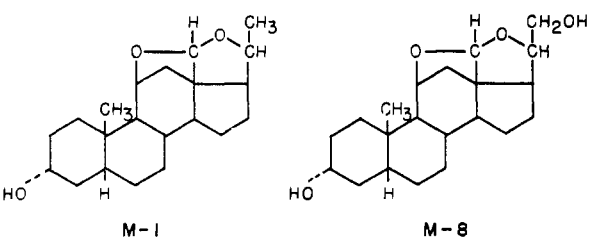

FIG. 1.—Two bicyclic acetal metabolites derived from aldosterone.

EXPERIMENTAL

All melting points were determined on a Kofler block and are corrected.

The infrared spectra were determined on a Model 221 Perkin-Elmer double-beam spectrometer. The nuclear magnetic resonance spectrum was determined on a Varian high-resolution nuclear magnetic resonance spectrometer.

All radioactivity measurements were made on a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co.). The radioactivity of tritium was counted at a photomultiplier high voltage of 1240 volts and discriminant settings 10 volts to 100 volts. Tritium and C^{14} together were measured according to the technique of Okita *et al.* (1957) at the same high voltage and discriminator

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settings of 10 to 100 volts and 100 to infinity volts. The scintillation solution was prepared by adding 7.11 g of PPO (2,5-diphenyloxazole) and 0.237 g of POPOP [1,4-bis-2(5-phenyloxazoyl-benzene] (Pilot Chemicals, Inc., Watertown, Mass.) to 2.37 liters of toluene (Mallinckrodt AR).

H^3-Aldosterone.—Seven hundred μg of *d*-aldosterone (isolated from bovine adrenals) was randomly tritiated according to the procedure of Wilzbach (1957) in the laboratories of the New England Nuclear Corporation. The labile tritium was exchanged for hydrogen with methanol and the methanol was removed under a stream of nitrogen. Immediately upon its return to this laboratory, the reaction mixture was chromatographed on paper in the Bush C system (900 ml toluene, 100 ml ethyl acetate, 500 ml methyl alcohol and 500 ml water) (Bush, 1951). Only one ultraviolet adsorbing zone, presumably aldosterone, was found on the paper. This zone was carefully outlined and cut out with the sacrifice of some of the material near the edge. The specific activity was determined by the micro blue tetrazolium reaction (Recknagel and Litteria, 1956). This process was repeated with the E_2B system (Eberlein and Bongiovanni, 1955) (*vide infra*) and then the Bush B_5 system (Bush, 1951) (1000 ml benzene, 500 ml water, 500 ml methyl alcohol). The aldosterone obtained from the last chromatogram (sample A) was used for metabolic studies.

In order to establish more rigorously the purity and identity of the radioactive material, an aliquot of about 500,000 cpm was removed from sample A and to it was added about 500 μg of *d*-aldosterone. The specific activity of the diluted sample was determined by the micro blue tetrazolium reaction. This material was chromatographed on paper, first in the Bush C system, then in the E_2B system, and the specific activities determined as before. The three specific activities recorded in Table I were in

TABLE I

PURIFICATION AND ESTABLISHMENT OF RADIOCHEMICAL HOMOGENEITY OF TRITIATED ALDOSTERONE

Chromatogram	Solvent System	Counts per Minute	Weight (μg)	Specific Activity (cpm/μg)
1	Bush C	7.0×10^5	*ca.* 170	*ca.* 4.1×10^6
2	E_2B	5.28×10^5	150	3.45×10^6
3	Bush B_5	2.74×10^5	105	2.61×10^6
460 μg of carrier added to 555,000 cpm		460		1200
4	Bush C	330,000	250	1320
5	E_2B	210,000	200	1050
				1190 ± 90 (8%)

The precision of the colorimetric measurement and of the counting was about ±10%.

agreement (±8%) within the order of the expected error of the measurements (±10%). Therefore sample A was assumed to be radiochemically pure *d*-aldosterone. The original 700 μg of *d*-aldosterone yielded 105 μg with a specific activity of 2.61×10^6 cpm/μg.

Administration of Aldosterone.—Synthetic *d*-aldosterone-21-monoacetate was administered orally in amounts varying from 2.5 to 50 mg a day over periods up to 2 weeks to six human subjects.

TABLE II

ADMINISTRATION OF ALDOSTERONE-H^3 TO HUMAN SUBJECTS

Subject	Amount Ingested (mg)	Amount Injected (cpm × 10^5)
A	97.5	8
B	35.0	2
C	25.0	2
D and E	160.0	12
F	160.0	16

Pool I was composed of the urine from subjects A, B, and C, volume 21,870 ml. Pool II was composed of the urine from subjects D and E, volume 18,400 ml. Pool III was the urine from subject F, volume 8040 ml. The substance ingested was *d*-aldosterone-21-monoacetate. The radioactive tracer injected was *d*-aldosterone-H^3 (see text). Subject A received 400,000 cpm of aldosterone-H^3 orally.

Tritiated *d*-aldosterone was administered orally or by intravenous injection in doses from 200,000 to 400,000 cpm according to the schedule shown in Table II. During the period of administration of the hormone and for 1 or 2 days thereafter all the urine was collected. The oral administration of the hormone in these quantities had no observable deleterious effects in any of these subjects.

Partition Column Chromatography.—All partition systems used in column chromatography were supported on Johns-Manville Celite 545 and are listed in Table III. The celite was first washed with 1 N HCl, then with sufficient water to remove the acid, and finally with methanol. Celite to be reused after chromatography was washed only with methanol. The stationary, polar phase of the solvent system was added to the celite in varying ratios from 1 g celite–1 ml solvent to 1 g celite–0.5 ml solvent. The material to be chromatographed was usually dissolved in the stationary phase and then mixed with the appropriate amount of celite.

TABLE III

PARTITION SYSTEMS EMPLOYED IN COLUMN CHROMATOGRAPHY

System	Components	g Celite/ml of Stationary 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	Iso-octane 0.5, *t*-butyl alcohol 0.25, water 0.45, methanol 0.1	2
F	Cyclohexane 1, methanol 0.85, water 0.15	1
G	Ethyl acetate 1, hexane 1, methanol 0.7, water 0.3	1
H	Methylene chloride 0.1, heptane 0.9, ethylene glycol 0.1	2
I	Ethyl acetate 0.33, hexane 0.67, methanol 0.35, water 0.15	1
J[a]	Methylcyclohexane 1, *N*,*N*-dimethylformamide 0.2	2
L	Ethyl acetate 1, hexane 1, methanol 0.6, water 0.4	1
M	Methylene chloride 0.5, methylcyclohexane 0.5, ethylene glycol 0.1	2
N	Methylene chloride 0.5, hexane 0.5, ethylene glycol 0.1	2
E_2B[b]	Iso-octane 0.5, *t*-butyl alcohol 0.25, water 0.45	2
E_4[b]	Iso-octane 0.5, *t*-butyl alcohol 0.225, water 0.05, methyl alcohol 0.225	2

[a] Touchstone and Kasparow (1960). [b] Eberlein and Bongiovanni (1955).

However, it was occasionally possible to dissolve the sample in a small volume of mobile phase and then to apply this solution to the column. The column was packed by adding a small charge of celite wet with stationary phase to the column and then applying moderate to heavy pressure with a tamping rod which perfectly fitted the column. Each charge when packed was 1 to 1.5 times as high as the diameter of the column. The volume of mobile phase retained by the column (hold-back volume, HBV) was approximately 1.5 times the weight of celite. It is possible to predict the volume of mobile phase (B) required for elution of a substance from the hold-back volume of the column, the volume of stationary phase (v), and the partition coefficient (K), by use of the relationship: $(B + 1)/B$ = number of HBV required for elution; $B = K$ (HBV/v) (Johnson, 1957).

Isolation of Aldosterone Metabolites.—After the administration of labeled aldosterone and unlabeled aldosterone-21-monoacetate, three urine pools were obtained (Table II). Each was separately treated with mammalian β-glucuronidase (Ketodase, Warner-Chilcott Co.) (600 units per ml) at 37° for 3 days at pH 5.0. After hydrolysis, each urine sample was extracted with an equal volume of ethyl acetate. The ethyl acetate was washed with 0.1 N NaOH until the washes were colorless, and then with water until neutral. The organic solvent was removed by vacuum distillation. Portions of the extracts from pools I and II, weighing 790 mg, 1410 mg, and 850 mg, were chromatographed on system A (Table III) on columns containing 240 g, 260 g, and 250 g of dry celite respectively. All of the extract from pool III was chromatographed on a 225-g column. Aliquots were removed from each fraction for quantification of blue tetrazolium-reducing substances and for determination of tritium. In Figure 2 a typical plot of the cpm of H^3 and the optical density at 520 mμ after blue tetrazolium reaction against fraction number is depicted. Three major zones were evident. The least polar zone (zone I), comprising the first half of the first hold-back volume, contained a large amount of radioactivity and much of the pigment. Zone II contained relatively little radioactivity, distributed in several small peaks, and a large amount of blue tetrazolium-positive material, presumably 3α,17α,21-trihydroxypregnane-11,20-dione (tetrahydrocortisone). The third and most polar zone (zone III), eluted in the third hold-back volume, contained more radioactivity than zone I. The exact amount of radioactivity was difficult to estimate at this point because of the quenching effect of pigments. The fractions comprising each zone were combined and the solvents removed under vacuum. A flow sheet outlining the isolation procedures used is given in Figure 3.

Zone I.

METABOLITES M-1, M-2, M-3 (FIG. 3).—M-1, M-2, and M-3 were isolated from pools I and II according to the procedure which follows. M-1, isolated from pool III, was subsequently obtained in crystalline form as described below.

The combined residue from zone I from urine pools I and II (weight 1.2 g) (*cf.* Fig. 2 and Table II) was chromatographed on a 200-g column in system G. The pigment present in this residue prevented an accurate determination of the tritium present. Fractions of 14 ml were collected and an aliquot was removed from each for counting. The combined residues from fractions 1 to 10 weighed 700 mg and contained an indeterminate quantity of tritium. The radioactivity could not be separated from the pigment in this residue by further chromatography. Fractions 19 to 41 had a combined weight of 480 mg and contained 100,000 cpm. The entire radioactive fraction was designated M-1, although the shape of the peak suggested a lack of

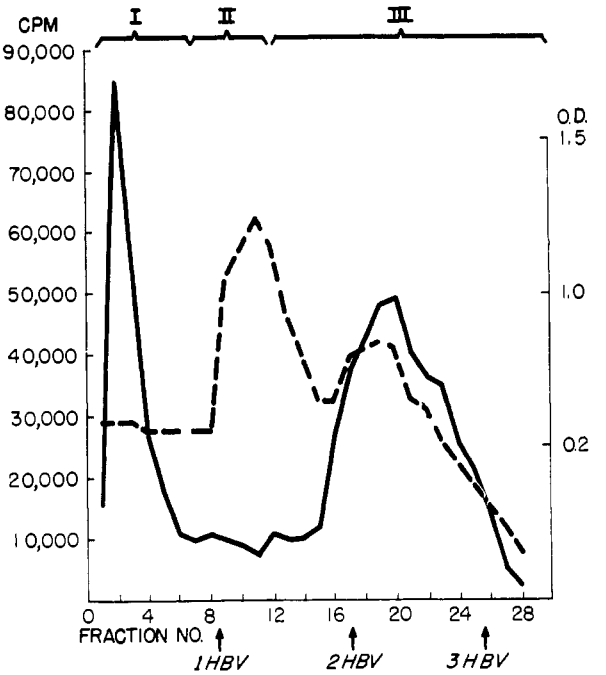

FIG. 2.—Typical partition chromatogram of the neutral extract in system A. The total cpm (———) and the optical density at 520 mμ of an aliquot after blue tetrazolium reaction (- - - - - - - - -) are plotted against fraction number. The hold back volumes (HBV) are indicated at the bottom and the zones (I–III) into which the eluates were divided are shown at the top.

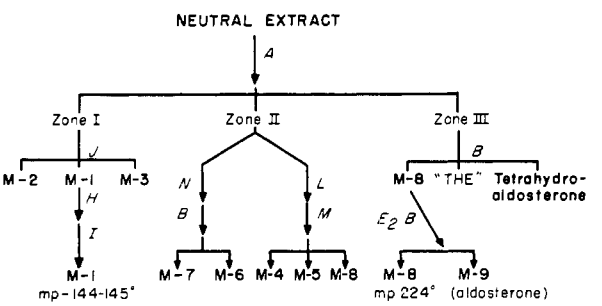

FIG. 3.—Simplified flow sheet for the isolation of aldosterone metabolites. "THE" refers to a nonradioactive, blue tetrazolium-positive material presumed to be tetrahydrocortisone. The letters A, B, E_2B, H, I, J, L, M, and N refer to the partition systems employed in the column chromatography, as given in Table III. Zones I, II, and III are defined in Figure 2 and in the text. M-1 through M-9 designate radioactive metabolites of aldosterone, as described in the text.

homogeneity. Subsequent chromatography of M-1 on a 50-g column in system H gave rise to a new, minor (5,000 cpm) radioactive component, M-2, present as a shoulder (less polar) on the M-1 peak. M-2 was not investigated further. The M-1 residue had a crude weight of 14 mg and contained 170,000 cpm. Chromatography of M-1 in system I reduced the weight to 7.9 mg and the counts to 80,000 cpm, but the unsymmetrical shape of the curve indicated lack of homogeneity. A new minor, more polar radioactive component, M-3, was clearly separated from M-1 in system J. The presence of *N,N*-dimethylformamide in this system made accurate counting impossible. M-3 was not investigated further because of the small amount available. M-1 was then chromatographed in system I, in which it appeared homogeneous. Attempts to obtain crystals from the combined fractions failed.

METABOLITE M-1.—The combined residues from zone I from the urine pool III (weight 477 mg) were chromatographed on a 100-g column in system J (*cf.* Fig. 3). Three distinct peaks were found by tritium determination, but the shape of the main peak, presumably M-1, suggested a lack of homogeneity. The slower of the minor peaks was in the same position as M-3, as previously described, and the faster-moving material was assumed to be M-2.

The residue presumed to contain M-1 was almost free of pigment and had 200,000 cpm. It was purified further by chromatography in system H on a 10-g column. Tritium determination revealed a symmetrical peak containing a total of 198,000 cpm in the expected hold-back volume. This is illustrated in Figure 4. The ethylene glycol present in the eluate was removed by chromatography in system I. Again, 159,000 cpm of tritium was distributed in a symmetrical peak in the expected hold-back volume.

The colorless oil (13 mg) left after removal of the solvents crystallized from ether-hexane to yield 8.5 mg of needles, m.p. 144–145°, with a specific activity of 16.8 cpm/μg (*vide infra*).

Zone II.

METABOLITES M-4, M-5 (*cf.* FIG. 3).—The combined residue from zone II from pools I and II (total weight = 470 mg) were chromatographed in system L on a 150-g column. The only peak which contained a significant amount of radioactivity was eluted at 3.3 hold-back volumes. Rechromatography of this material in system M afforded two poorly separated components eluted at 4.9 and 5.3 hold-back volumes, designated M-4 and M-5 respectively. A third substance, eluted later, was identified by infrared spectroscopy as M-8 (*vide infra*). The quantities of M-4 and M-5 were insufficient to permit further study.

METABOLITES M-6 AND M-7 (FIG. 3).—Zone II from pool III (114 mg), was chromatographed on a 50-g column in system N. The only radioactive material eluted from this column was found at 10.8 hold-back volumes. A total of 20,000 cpm was recovered and rechromatographed in system B, in which a new minor component, M-7, was eluted at 0.9 hold-back volumes, and a new major component, M-6 was eluted at 1.8 hold-back volumes. Attempts to crystallize M-6 at this point did not succeed, nor could crystals be obtained after chromatography in system D. Although M-6 is similar in its chromatographic properties to M-8, it is slightly but definitely less polar. On system B it is eluted at 1.5 hold-back volumes, whereas M-8 is eluted at 2.2 hold-back volumes; and on system D it is eluted at 2.0 hold-back volumes, whereas M-8 is eluted at 3.8 hold-back volumes. Therefore, it can be assumed that M-6 and M-8 are different compounds.

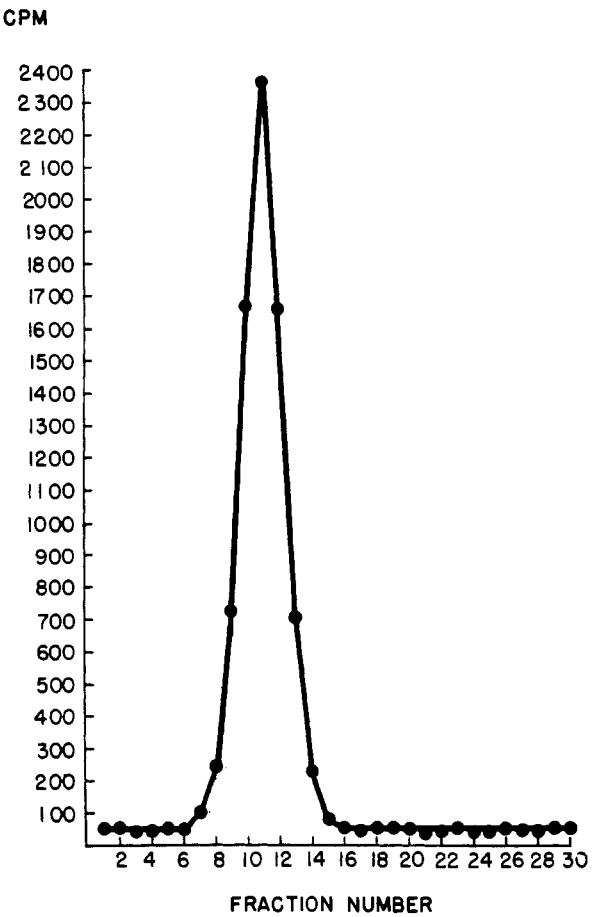

FIG. 4.—Partition chromatogram of M-1 from pool III on system H (*cf.* Table III). The cpm of H^3 in a $^1/_{25}$th aliquot are plotted against fraction number. The peak fraction was eluted at 3.7 hold-back volumes.

The minor metabolites, M-4 and M-5, were not isolated from pool III, nor was any evidence of M-6 and M-7 found in pools I and II.

Zone III.

METABOLITES M-8 AND M-9 (FIG. 3).—Zone III consisted mainly of tetrahydroaldosterone. No significant radioactivity was eluted later than zone III, and virtually none could be removed from the column by washing with methanol. Zone III was rechromatographed in system B, in which the tetrahydroaldosterone was completely separated from traces of "tetrahydrocortisone" and from a small amount of a more rapidly migrating radioactive substance (*cf.* Fig. 3). Further description of the isolation and characteristics of tetrahydroaldosterone will be given in another report. The

fractions containing the other radioactive material were combined and designated M-8. M-8 from pools I and II were pooled and chromatographed on system C and again on system B. In all of these chromatograms M-8 appeared homogeneous. However, acetylation of a portion of M-8 with C^{14}-labeled acetic anhydride, followed by chromatography on paper in the E_4 system (Table III) (Eberlein and Bongiovanni, 1955), afforded two substances, each containing both tritium and C^{14}. One of these absorbed ultraviolet light maximally at 239 mμ and reacted with the blue tetrazolium reagent at the normal rate. This material was designated M-9 acetate. The second substance, less polar, did not absorb UV light nor did it reduce blue tetrazolium. It was designated M-8 acetate. Resolution of the unacetylated M-8 and M-9 was achieved by column chromatography in the E_2B system, in which M-8 was eluted in the first hold-back volume and M-9 in the third hold-back volume.

METABOLITE M-8.—Removal of the solvents from the fractions comprising the first hold-back volume of the E_2B column on which M-8 and M-9 were separated led to the recovery of a few (<100 μg) crystals melting at 224°. M-8 was also isolated from pool III, as described above, by use of partition systems A, B, and E_2B successively. Crystalline material weighing 1.5 mg was found in the tubes containing most of the radioactivity; this was recrystallized from acetone-ligroin, and again from acetone. The product melted at 224–225°. This substance could also be crystallized from methanol–diethylether and methylene chloride–*n*-hexane.

METABOLITE M-9.—The chemical (oxidation by the blue tetrazolium reagent) and chromatographic properties of M-9 and M-9 acetate suggest that these compounds were aldosterone and aldosterone diacetate respectively. Both absorbed ultraviolet light at 239 mμ, and the infrared spectrum of M-9 in chloroform was similar to that of aldosterone (Simpson *et al.*, 1955). The infrared spectrum of M-9 acetate was identical with the spectrum of authentic aldosterone diacetate prepared from *d*-aldosterone-21-monoacetate. A total of 500 μg of aldosterone was isolated from pools I and II, and an additional 500 μg was isolated from pool III.

STRUCTURAL STUDIES

Determination of Acetylatable Hydroxyl Groups.—M-1, M-8, and M-9 were acetylated with C^{14}-labeled acetic anhydride in pyridine at room temperature for 18 hours. Desoxycorticosterone was acetylated under the same conditions and the resultant monoacetate was recrystallized until the crystals from two successive crystallizations and the final mother liquor had the same specific activity. Thus the specific activity of the acetic anhydride could be expressed in terms of the specific activity of the desoxycorticosterone acetate prepared from it. The number of acetylatable hydroxyl groups in a given metabolite is equal to the specific activity of its acetate with respect to C^{14} divided by that of desoxycorticosterone acetate. The specific activity with respect to C^{14} of M-1, M-8, and M-9 acetates was determined from the product of the ratio of C^{14} cpm to H^3 cpm for the acetate and the specific activity of the metabolite with respect to H^3: $(\text{cpm } C^{14}/\text{cpm } H^3)_{\text{acetate}} \times (\text{cpm } H^3/\text{wt})_{\text{metabolite}} = \text{cpm } C^{14}/\text{wt} = \text{specific activity of acetate}$.

The weight parameter of the specific activity with respect to H^3 for M-1 was determined directly. The weight of M-9 was estimated from its ultraviolet absorbance (assuming $E_{239} = 15{,}000$) and by quantitation with the blue tetrazolium reagent, with aldosterone monoacetate used as the standard. Because the large amount of aldosterone administered in this study masked any endogenous contribution to the weight of the metabolites, the specific activities with respect to H^3 of all of the metabolites isolated from a given urine pool would be expected to be about the same. The specific activities of M-9 and of tetrahydroaldosterone, both isolated from the same pool, were identical. Therefore it was assumed that the specific activity of M-8 from the same pool was equal to that of M-9.

METABOLITE M-1.—M-1 did not react with the blue tetrazolium reagent and did not absorb UV light in the 240 mμ region. Furthermore, M-1 did not produce formaldehyde on reaction with HIO_4 according to the procedure of Wilson (1953). Acetylation with C^{14} acetic anhydride gave an acetate which was purified by chromatography in system F. Its specific activity was 1.16 times that of desoxycorticosterone acetate prepared from the same C^{14} acetic anhydride, and it is therefore a monoacetate. Its infrared spectrum, shown in Figure 5, was remarkable in that it demonstrated the absence of absorption in the carbonyl region between 1800 and 1600 cm^{-1}. The infrared spectrum of the monoacetate, shown in Figure 6, confirmed the presence of an acetoxy group (*cf.* bands

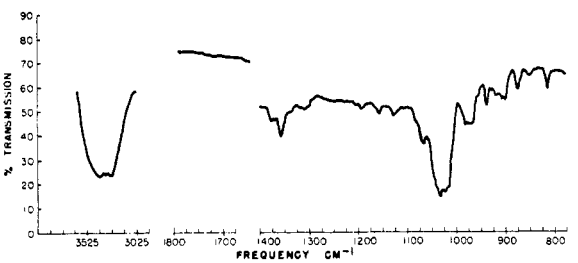

FIG. 5.—Infrared spectrum of M-1 (*ca.* 1 mg) in KBr.

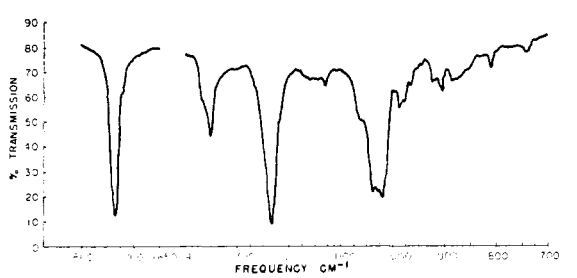

FIG. 6.—Infrared spectrum of M-1 acetate (*ca.* 1 mg) in CS_2 using a 1-mm micro cell compensated with a 1-mm macro cell.

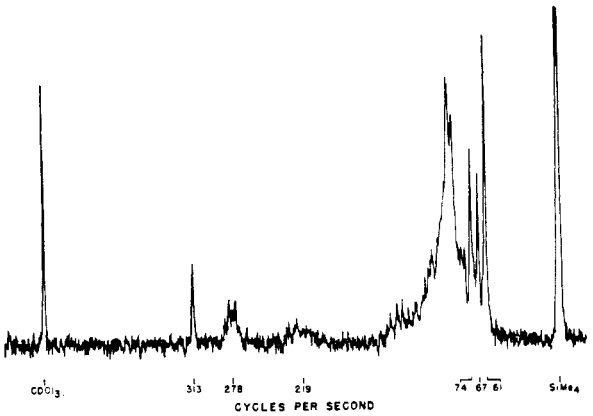

FIG. 7.—Nuclear magnetic resonance spectrum of M-1 (4.5 mg) in $CDCl_3$. $SiMe_4$ was added as a reference compound.

at 1735 and 1240 cm^{-1}). The absence of a conjugated ketone and the lack of reactivity toward blue tetrazolium were consistent with the infrared spectra of M-1 and M-1 acetate.

A high-resolution nuclear magnetic resonance spectrum of M-1 (Fig. 7) was obtained at 60 megacycles in a $CDCl_3$ solution. Tetramethylsilane was added to the solvent to serve as an internal reference, and the peak positions were measured relative to this reference in cycles per second. Increasing frequency in this system corresponds to a decreasing applied magnetic field. The nuclear magnetic resonance spectrum of M-1 revealed the presence of at least three, and probably not more than four, protons bonded to carbon bearing oxygen (313, 278, and 219 cps), an angular methyl group at C_{10} (61 cps), and a second methyl group attached to a carbon atom with a proton (67 and 74 cps). The sharpness of the peak at 313 cps and the large size of the chemical shift indicate the presence of a carbon atom attached to two oxygen atoms.

KETO-LACTONE ACETATE FROM M-1—Two mg of M-1 acetate was oxidized with 2% chromic acid acid solution in 90% acetic acid. About 3 equivalents of CrO_3 were added in the course of 2 hours. A 4th did not seem to be consumed during a night at room temperature. An aqueous solution of the reaction mixture was extracted with ether, and the ether washed first with dilute $NaHCO_3$, then with water until neutral. After evaporation of the ether, a crystalline product, m.p. 170–175°, was obtained. Recrystallization raised the melting point first to 183–193° and finally to 203–206°. The infrared spectrum (Fig. 8) gave no evidence of a hydroxyl function, but did show carbonyl absorption at 1780 cm^{-1} (γ-lactone), 1735 cm^{-1} (ester), and 1712 cm^{-1} (ketone), and a single band at 1240 cm^{-1} (acetoxy).

DIKETO-LACTONE FROM M-1.—One and one half mg of M-1 was oxidized as above by the addition of approximately 5 equivalents of CrO_3. The product, isolated as described above, was recrystallized from ether-methanol and melted at 220–223°. The melting point was unchanged after successive recrystallizations from ether-methanol and from methylene chloride–hexane, as well as after rechromatography in the E_4 system and subsequent re-

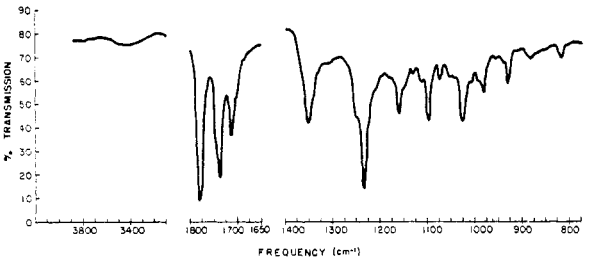

FIG. 8.—Infrared spectrum of the keto-lactone acetate prepared from M-1 acetate by oxidation with chromic acid. About 1 mg of the compound was dissolved in CS_2 and the spectrum determined in a 1-mm micro cell compensated with a 1-mm macro cell.

crystallization from methylene chloride–hexane and from ether-methanol. Its infrared spectrum was identical with that of authentic[1] 11β-hydroxy-3,20-diketopregnan-18-oic lactone (18 → 11) (Velluz *et al.*, 1960), as shown in Figure 9. The synthetic

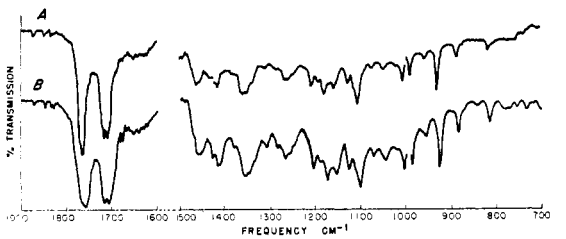

FIG. 9.—*A*, Infrared spectrum of the diketo-lactone (*ca.* 10 μg) prepared from M-1 by chromic acid oxidation. A micro KBr pellet (diameter 1.5 mm) was used with a sample beam condensing unit. *B*, The spectrum of authentic diketo-lactone determined in KBr as for *A*.

lactone melted at 234–237°, with an apparent change in crystal form from plates to needles at about 220°. Recrystallization of the authentic sample from ether-methanol and from methylene chloride–hexane did not alter the melting point. A mixture of the synthetic sample with the lactone obtained from the metabolite melted at 220–230°, with the bulk of the material melting at 228–230°. The melting point of the lactone from the urinary metabolite could not be raised by recrystallization from diethylether-methanol in the presence of a seed of the synethetic lactone.

METABOLITE M-8.—The infrared spectrum of M-8 (Fig. 10) revealed the presence of absorption

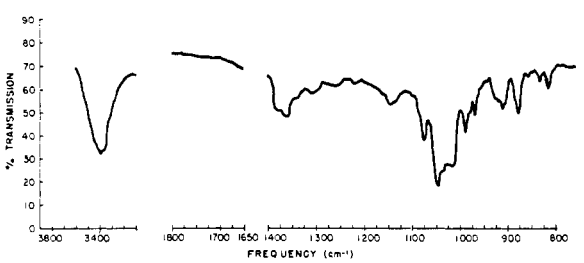

FIG. 10.—Infrared spectrum of M-8 (*ca.* 10 μg) in a micro KBr pellet with a beam condensing unit.

[1] A sample of this substance was provided through the generosity of Professor Léon Velluz and Mr. François G. Robinet of UCLAF-Roussel, Paris.

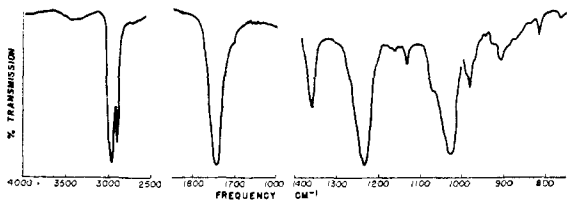

FIG. 11.—Infrared spectrum of M-8 acetate (*ca.* 200 μg) in CS_2, with a 0.2-mm micro cell and a compensation cell.

bands in the hydroxyl region and the absence of any absorption bands in the carbonyl region between 1800 and 1600 cm^{-1}. The C^{14}/H^3 ratios of M-8 acetate from pools I and II, after chromatography in system F, were the same as those of aldosterone diacetate isolated from the same pools and prepared from the same C^{14}-acetic anhydride. Therefore, M-8 also forms a diacetate, the infrared spectrum of which shows carbonyl absorption only at 1735 cm^{-1} and a single intense band at 1240 cm^{-1} (Fig. 11). Crystalline M-8 did not absorb ultraviolet light in the region of 240 mμ, it did not react with blue tetrazolium, either normally or slowly, and it did not produce formaldehyde on treatment with periodate (Wilson, 1953).

KETOL-LACTONE DIACETATE FROM M-8.—One and one half mg of M-8 diacetate was oxidized with approximately 3 equivalents of 2% CrO_3 solution in 90% acetic acid. The product was isolated from the reaction mixture by the procedure described above for M-1 keto-lactone acetate. The crude material thus obtained was chromatographed in the E_4 system on 10 g of celite. Upon removal of the solvents from the combined fractions containing the bulk of the radioactivity, which had been eluted in the 6th hold-back volume, a blue tetrazolium–positive crystalline product, m.p. 162–164°, was obtained. The infrared spectra of this substance and that of 3α,21-diacetoxy-11β-hydroxy-20-ketopregnan-18-oic (18 → 11) lactone (shown in Fig. 12), m.p. 162–164°, were identical. The melting point of a mixture of both samples was not depressed. The latter compound was prepared from tetrahydroaldosterone diacetate by chromic acid oxidation. The isolation of tetrahydroaldosterone, the preparation of its diacetate, and the oxidation of the diacetate to the lactone will be described in detail in a report now in preparation.

RESULTS AND DISCUSSION

Evidence has been presented for the occurrence of ten urinary metabolites of aldosterone excreted as glucuronosides. The major metabolite is tetrahydroaldosterone, and another urinary product has been identified as unchanged aldosterone. This investigation confirms a previous report that small amounts of aldosterone are excreted as a glucuronoside conjugate (Flood *et al.*, 1961). In this investigation eight previously unknown compounds were detected. Two of these were isolated in crystalline form and were identified, whereas the remaining six compounds were present in such small amounts that crystallization and further characterization were not possible. All the new metabolites isolated by procedures described in this paper are less polar than tetrahydroaldosterone. Four of these (M-1, M-8, aldosterone, and M-6) were isolated in relatively substantial amounts, although tetrahydroaldosterone was by far the most abundant metabolite. These data are summarized in Table IV, wherein is shown the amount of each metabolite isolated from pool III, together with the percentage of the injected tritiated aldosterone which appears in each of these metabolites. Since the approximate specific activity of the administered hormone

$$\frac{\text{(cpm injected)}}{\text{(mg ingested)}}$$

was 10,000 cpm/mg and the metabolites had a specific activity of about 15,000 cpm/mg, it is probable that some of the ingested steroid was not absorbed. Thus, it is not possible to calculate the per cent conversion of the hormone to its metabolite on the basis of the weight ingested. Thirty-two per cent of the weight of aldosterone fed was recovered as the five most abundant metabolites, whereas 39% of the radioactivity intravenously administered was recovered in these same metabolites. Since these figures do not take operational losses into account, they represent the minimum amounts of each substance originally present in the urine as well as the minimum conversion of aldosterone to these metabolites. Furthermore, since operational losses can be reasonably assumed to be about the same for all substances isolated, ratios of each of these metabolites to the others are probably correct as given. However, these ratios may not necessarily be those which obtain for metabolites of endogenous origin.

Each of the other five metabolites (M-2, M-3, M-4, M-5, and M-7) represented less than 1% of the injected radioactivity. Their presence in detectable amounts in a given urine sample may depend on the metabolic characteristics of the individual subject. Thus M-4 and M-5 were isolated only from pools I and II and M-6 and M-7 only from pool III, although M-2 and M-3 were isolated in every case. The origin of these five radioactive substances is unknown at present, because the possibility that some, or all, of them were derived from undetectable radioactive impurities present in minute amounts in the radioactive aldosterone administered cannot be excluded.

It has been reported (Nowaczynski *et al.*, 1956; Sandor *et al.*, 1960) that 21-hydroxy-18-oxo-Δ^4-pregnene-3,11,20-trione (11-dehydroaldosterone) is a urinary metabolite of the hormone. However, the criteria upon which both the structure of this isolated material and its relation to aldosterone were based were insufficient. In direct contradiction to these reports, this metabolite was not isolated in the course of these experiments, although it might be expected that any metabolite present in significant amounts would have been detected after the administration of large amounts of aldosterone.

On the basis of the evidence already presented, it is possible to propose structures for the two crystalline metabolites, M-1 and M-8.

Although M-1 is a metabolite of the $C_{21}O_5$ corticosteroid, aldosterone, its partition coefficient is

TABLE IV

AMOUNTS OF METABOLITES ISOLATED AFTER THE ADMINISTRATION OF ALDOSTERONE-H³ TO SUBJECT F[a]

Metabolite	Amount Isolated (mg)	% Conversion[b]
Tetrahydroaldosterone	40	28.0
M-1	8.5	8.0
M-8	1.5	1.4
M-6	1	1.2
Aldosterone	0.5	0.5
	51.5	39.1[c]

[a] *Cf.* Table II and text. [b] % Conversion = cpm isolated × 100/cpm injected i.v., *cf.* text. [c] Compare with 32% conversion to these metabolites on a weight basis: mg metabolites isolated × 100/mg aldosterone ingested = 51.5 × 100/160.

comparable to that of testosterone. It contains no carbonyl group and only one acetylatable hydroxyl function. It does not reduce the blue tetrazolium reagent, nor does it produce formaldehyde on oxidation with periodic acid. Hence, it is evident that the side-chain present in M-1 contains neither a glycol nor a ketol grouping. These unusual properties, together with the absence of carbonyl absorption in the infrared between 1800 and 1600 cm^{-1}, indicate that the oxygenated functions originally present in aldosterone at C_{11}, C_{18}, C_{20}, and C_{21} are present in this metabolite, bound in some unreactive form, possibly as an acetal.

Further support for this view came from the nuclear magnetic resonance spectrum, which revealed the presence of two methyl groups. One of these was, as expected, at C_{10} (61 cps). The other methyl group was not originally present in aldosterone. This group is characterized by a pair of peaks found at 67 and 74 cps. The sharpness of these peaks indicates that they arise from a methyl group. The 7-cps separation of the peaks suggests that this second methyl group is attached to a carbon atom which has one proton. These findings can be reasonably interpreted to indicate the presence of a Cm_{21} ethyl group. The spin-spin coupling between the methyl group and the adjacent proton results in a doubling of the methyl resonance as observed.

The sharpness of the peak at 313 cps and the large size of the chemical shift are strongly indicative of a proton on a carbon atom bearing two oxygens. The proton on C_{20} would be split by spin coupling to the neighboring methyl group and to the proton on C_{17}, and this might account for the shape of the 278-cps peak. These facts reinforce the suggestion that the second methyl group is on C_{20}, which, itself, is linked to one proton and one oxygen atom. The peak at 313 cps would be assigned to the proton on C_{18}, the peak at 278 cps would represent the proton on C_{11} with the resonance of the proton on C_{20} superimposed on it, and the peak at 219 cps would arise from the proton on C_3. Furthermore, that there are four protons linked to oxygen-bearing carbon atoms is indicated by the integrated intensities of the appropriate peaks. The peaks at 313 and 219 cps each represent one proton, and the peak at 278 cps represents two protons.

Thus, data from both nuclear magnetic resonance and infrared spectra support the view that the 11, 18, and 20 oxygen functions of M-1 must be in acetal form (see Fig. 1). The occurrence in M-1 of a 21-methyl group, which in the precursor, aldosterone, was present as a 21-CH_2OH group, is consistent with the failure of M-1 to react with periodic acid and the blue tetrazolium reagent and with the formation of a monoacetate which shows no further hydroxyl absorption in the infrared.

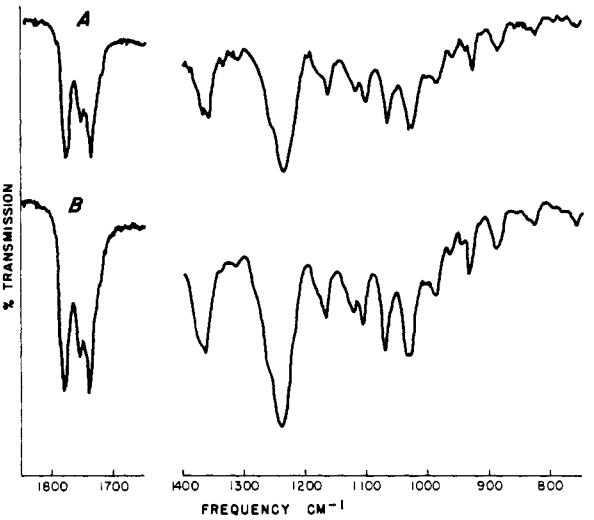

FIG. 12.—*A*, Infrared spectra of the product of chromic acid oxidation of M-8 diacetate. *B*, Infrared spectrum of 3α,21 - diacetoxy - 11β - hydroxy - 20 - ketopregnan - 18 - oic (18 → 11) lactone prepared from tetrahydroaldosterone (*cf.* text). The region 1850 to 1650 cm^{-1} was determined in CS_2 solution (containing *ca.* 200 μg) with a 0.2-mm microcell and a variable compensating cell. The fingerprint region (1400–800 cm^{-1}) was determined in KBr (containing *ca.* 10 μg, 1.5-mm diameter pellet) with a sample beam condensing unit. The carbonyl region was best resolved in CS_2 solution, whereas the fingerprint region was more detailed when determined in KBr.

Confirmation of this structure was achieved by oxidation of the metabolite M-1 to the known keto-lactone, 11β-hydroxy-3,20-diketopregnan-18-oic-(18 → 11) lactone. The infrared spectra of the synthetic compound and the product derived from M-1 were identical, as shown in Figure 9. Although the authentic keto-lactone had a higher melting point than the derivative obtained from M-1, a mixture of both substances melted between the melting points of the individual compounds. This behavior might be expected if the low melting point of the derivative of the metabolite were due to the presence in the sample of an impurity that was difficult to remove and whose presence was not evident from the infrared spectrum. If this were true, the keto-lactone from M-1 might be expected to melt at the temperature at which the synthetic keto-lactone undergoes a change in the crystal structure. In spite of this unexplained behavior in melting, the two substances can be considered identical because of the exact correspondence between their spectra. Thus, the bicyclic acetal, 3α-hydroxypregnan, (11β-18), (18–20) dioxide, is the only structure for M-1 consistent with the available data.

The stereochemistry of this metabolite differs from that of aldosterone in that four new asymmetric centers have been introduced. Of these, C_5 is the one for which the stereochemistry is best known because it has been established by direct comparison with a known 5β-compound. From this and from the infrared data, which indicate that the 3-acetoxy group and the 5-hydrogen of the monoacetate and also of the lactone monoacetate are in a *trans* relationship (Jones and Herling, 1956), it follows that the 3-hydroxyl group is α. The most abundant metabolite formed by the *in vivo* conversion of a 20-ketone to a 20-alcohol is usually a product of the 20α-series. This fact strongly suggests the α-configuration for the C_{20}-O- bond in M-1. No evidence has been adduced for the stereochemistry at C_{18}. Inspection of atomic models does not eliminate any of the four possible stereoisomers about C_{18} and C_{20}, but does demonstrate the requirement that the 11-oxygen function have the β-configuration.

The second crystalline metabolite, M-8, has a partition coefficient similar to that of its precursor, aldosterone. Like M-1, it lacks a carbonyl function and is unreactive toward the blue tetrazolium reagent and periodic acid in the formaldehydrogenic test. However, it has two hydroxyl groups, both of which can be acetylated. Oxidation of the diacetate with chromic acid in acetic acid gave rise to a blue tetrazolium–positive product whose infrared spectrum (Fig. 12) indicated the presence of a γ-lactone and a 21-acetoxy-20-keto group. This lactone proved identical with 3α,21-diacetoxy-11β-hydroxy - 20 - ketopregnan - 18 - oic - (18 → 11) lactone, prepared from tetrahydroaldosterone, as evidenced by melting point, mixed melting point, and infrared spectra.

On the basis of the above-mentioned physical and chemical properties, it is possible to propose a structure for this metabolite. Chromic acid oxidation of the diacetate of M-8 yields a γ-lactone, as does chromic acid oxidation of M-1. This fact may be interpreted as evidence that both metabolites possess a bicyclic acetal structure (-C_{11}-O-C_{18}H-O-C_{20}-). Whereas M-1 acetate yielded under these oxidative conditions an acetate of a *keto*-lactone, M-8 diacetate gave rise to an acetate of a *ketol*-lactone. Unlike M-8, the ketol-lactone is blue tetrazolium–positive, and this fact, added to the evidence from infrared spectroscopy, indicates that the formation of a ketol-lactone has resulted from the oxidative cleavage of a bicyclic acetal bearing a primary acetoxy group on C_{21}. Therefore the structure (Fig. 1) 3α,21-dihydroxypregnane-(11β-18), (18–20) dioxide is proposed for M-8. This metabolite then differs from M-1 in having an additional hydroxyl group at C_{21}. Whether the two metabolites have the same configurations at C_{18} and C_{20} is undetermined at present, although, for the same reasons as those advanced in the case of M-1, it is likely that the C_{20}-O- bond in M-8 is α-oriented.

Additional support for the -C-O-CH-O-C structures for M-1 and M-8 comes from infrared spectroscopy. It is well known that simple aliphatic ethers exhibit a very strong absorption band in the region of 1150–1060 cm^{-1} which is associated with the C-O-C structure. A study of a series of tetrahydrofuran derivatives has shown that these compounds absorb strongly between 1075–1100 cm^{-1} (Barrow and Searles, 1953). Compounds containing a C-O-C-O-C structure give spectra possessing multiple bands in this region. Bergmann and Pinchas (1952) studied eighteen ketals and acetals, many of which were cyclic dioxolanes, and identified a series of four bands (1158–1190 cm^{-1}, 1124–1143 cm^{-1}, 1063–1098 cm^{-1} [strongest], and 1038–1056 cm^{-1}) which they regarded as characteristic of these C-O-C groupings. Tschamler and Leutner (1952) have also observed that dioxanes and dioxolanes have multiple band structures in this frequency range. The precise number of characteristic frequencies produced by these complicated cyclic ethers has, however, not been clearly defined (LaGrange and Mastagli, 1955; Tschamler and Leutner, 1952).

Multiple absorption bands in the region between 1075–1010 cm^{-1} were found in the spectra of both M-1 and M-8 as well as in those of their acetates. In M-1 and M-8 the C-O stretching absorptions of the hydroxyl groups are also present, and these make it difficult to distinguish which of the many bands is associated with an oxide. This is not so for M-1 acetate and M-8 diacetate, in whose spectra the presence of the medium absorption bands at about 1060 cm^{-1} and the very strong complex of bands in the region of 1030–1010 cm^{-1} is consistent with the C-O-C-O-C structure. The occurrence of these bands at about 50 cm^{-1} below the frequency expected for five-membered cyclic ethers (Barrow and Searles, 1953; Bergmann and Pinchas, 1952) could be due to ring strain, which is known to cause a shift to lower frequencies. Strain in the bicyclic acetal systems in these metabolites may be present, in part, because C_{12} cannot be coplanar with the five-membered acetal ring composed of C_{11}, C_{12}, C_{13}, C_{18}, and the oxygen atom. Tetrahydrofuran is considered to be planar and unstrained, but, in the metabolites, one atom of the heterocyclic ring, C_{12}, is distorted out of the plane by virtue of the rigidly held chair form of ring C. Oxidation of the acetoxy derivative of either metabolite to the corresponding 18→11β lactone results in the disappearance of the strong absorption bands in the region between 1075 and 1010 cm^{-1}.

Thus the two new metabolites differ from their progenitor in that they both possess a bicyclic acetal structure rather than the cyclic hemiacetal present in aldosterone. The acetal groups on both are made possible by the fact that the 20-carbonyl group of the hormone has probably first been converted metabolically to a secondary hydroxyl group, which then reacts with the neighboring hemiacetalic hydroxyl group on C_{18} by elimination of water. While the possibility that the elimination reactions resulting in the formation of the acetals occur during the isolation procedures has not been excluded, it seems unlikely. The isolation techniques employed consisted merely of glucuronidase hydrolysis of the urine (at pH 5), extraction of the liberated metabolites by ethyl acetate, and resolution of the resulting organic, extractable

mixture into its components by a series of partition chromatograms with celite used as a support. Evaporation of all organic solvents was carried out *in vacuo* on a flash evaporator at 45°. None of these procedures is known to result in the elimination of the elements of water. It is, of course, possible that the close proximity of a 20-hydroxyl and the slightly acidic hemiacetalic hydroxyl group on C_{18} greatly facilitates this elimination and that even conditions ordinarily considered mild could bring about such a reaction. On the other hand, the numerous examples of the *in vivo* formation of acetals, particularly glycosides, provide ample precedence for the existence of metabolic products such as M-1 and M-8. Especially noteworthy is the analogous bicyclic ketal structure which characterizes the naturally occurring steroidal sapogenins.

The structure of M-1 illustrates another unusual feature of steroid catabolism. Although it is derived from a hormone bearing a hydroxymethyl group on C_{20}, the corresponding 20-substituent in the metabolite is a methyl group. The conversion of 21-hydroxylated steriods to 21-deoxy metabolites has previously been demonstrated (*e.g.*, desoxycorticosterone → pregnane-3α, 20α-diol [Hoffman *et al.*, 1943]; 11-dehydrocorticosterone [compound A] → 11-keto-pregnane-3α, 20α-diol [Mason, 1948]); but in each case it is a relatively minor pathway. In contrast, this metabolic transformation appears to be a significant pathway for the catabolism of aldosterone (Table III). All the examples to date reveal that those 21-deoxy metabolites which are formed from 21-hydroxy-20-keto precursors have always been isolated as the 20-alcohols. It is interesting to note that all four of the urinary metabolites of aldosterone characterized so far retain the functional groups at C_{11} and C_{18} in the same state of oxidation as those present in the hormone.

Acknowledgments

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