REFERENCES

Beal, P. F., and Pike, J. E. (1960), *Chem. Ind. (London)*, 1505.

Eberlein, W. R., and Bongiovanni, A. M. (1955), *Arch. Biochem. Biophys. 59*, 90.

Heusler, K., Kalvoda, J., Meystre, C., Wieland, P., Anner, G., Wettstein, A., Cainelli, G., Arigoni, D., and Jeger, O. (1961), *Helv. Chim. Acta 44*, 502.

Heusler, K., and Wettstein, A. (1962), *Helv. Chim. Acta 45*, 347.

Jackman, L. M. (1959), Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, New York, Pergamon, pp. 115–119.

Karplus, M. (1959), *J. Chem. Phys., 30*, 11.

Kelly, W. G., Bandi, L., and Lieberman, S. (1962b), *Biochemistry 1*, 792.

Kelly, W. G., Bandi, L., and Lieberman, S. (1963), *Biochemistry 2*, 1249.

Kelly, W. G., Bandi, L., Shoolery, J., and Lieberman, S. (1962a), *Biochemistry 1*, 172.

Okita, G. J., Kabara, J. J., Richardson, F., and LeRoy, G. V. (1957), *Nucleonics 15*, 111.

Schmidlin, J., and Wettstein, A. (1962), *Helv. Chim. Acta 45*, 331.

Shoolery, J. N., and Rogers, M. T. (1958), *J. Am. Chem. Soc. 80*, 5121.

Velluz, L., Muller, G., Bardoneschi, R., and Poittevin, A. (1960), *Compt. Rend. 250*, 725.

Wieland, P., Heusler, K., and Wettstein, A. (1961), *Helv. Chim. Acta 44*, 2121.

Isolation and Characterization of Human Urinary Metabolites of Aldosterone. V. Dihydroaldosterone and 21-deoxytetrahydroaldosterone*

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The nature of the metabolites of aldosterone excreted in the urine following the oral administration of a large quantity of the tritium-labeled hormone to normal human subjects is the subject of this and preceding papers. This paper reports the isolation and characterization of two metabolites, 4,5β-dihydroaldosterone (11β,21-dihydroxy-18-oxopregnane-3,20-dione) and 21-deoxytetrahydroaldosterone (3α,11β-dihydroxy-18-oxopregnan-20-one), thus bringing to eight the number of aldosterone metabolites identified in this study. In addition a description of the partial characterization of four unidentified metabolites is given.

Previous reports (Kelly *et al.*, 1962a,b, 1963) from this laboratory have described the isolation from human urine of a number of metabolites of administered aldosterone, as well as the characterization of these metabolites by degradation, and in some cases synthesis. This report concerns the isolation and proof of structure of two additional metabolites, namely, dihydroaldosterone (11β,21-dihydroxy-18-oxopregnane-3,20-dione), M15, and 21-deoxytetrahydroaldosterone (3α,11β-dihydroxy-18-oxopregnane-20-one), M6. In addition, the isolation of several previously unknown metabolites is reported; however, because of the small amounts isolated, it was not possible to obtain sufficient data for the determination of the structure of these metabolites. The method of isolation of the metabolites is schematically illustrated in Figure 1. The proofs of structure of M15 and M6 are presented in Figures 2 and 3, respectively. M15 was characterized by oxidizing it with periodic acid to the known 3-ketolactone, M12-5, which had been prepared from tetrahydroaldosterone (Kelly *et al.*, 1962b). The structure of M6 was established by means of its conversion to the diacetate M6-1, which was in turn characterized by its spectroscopic properties and by its oxidation with chromic acid to the ketolactol monoacetate M6-2.

EXPERIMENTAL

All melting points were obtained using a Kofler block and are corrected. Infrared spectra were determined on a Perkin Elmer Model 221 spectrometer. Radioactivity as tritium and carbon-14 was counted as described previously and the partition chromatography was carried out as in previous studies (Kelly *et al.*, 1962a,b, 1963; Okita *et al.*, 1957). The partition systems used are given in Table I.

Isolation of Metabolites

A flow sheet summarizing the isolation of the metabolites is presented in Figure 1. The preparation of a neutral extract from the urine of human subjects to whom several hundred mg of tritium-labeled *d*-aldosterone-21-monoacetate had been orally administered, and the chromatographic separation of this extract into zones I, II, and III have been described (Kelly *et al.*, 1962a,b).

The separation of M6 from M7, M8, M9, M10, M11, and M12 by chromatographic analysis of zone III in system B has been described in preceding papers of this series (Kelly *et al.*, 1962a,b). Zone II from the chromatogram on system A was chromatographed in system B and a radioactive substance was eluted in 1.5 hold-back volumes. This substance, presumed to be M6, was combined with M6 from zone III. Rechromatography of the combined M6 in system B gave a single radioactive peak which was eluted in 1.5 hold-back volumes. However, M6 was separated into four components upon chromatography in system M. These substances were eluted at 2.4, 3.2, 5.2, and 8.0 hold-back volumes and were designated M13, M14, M15, and M6, respectively. Although M14 and M15 were poorly separated in this system, they were later separated easily, as described. M6 was rechromatographed in system D from which it was eluted in 2.0 hold-back volumes, and then in system W from which it was eluted in 4.0 hold-back volumes. No other

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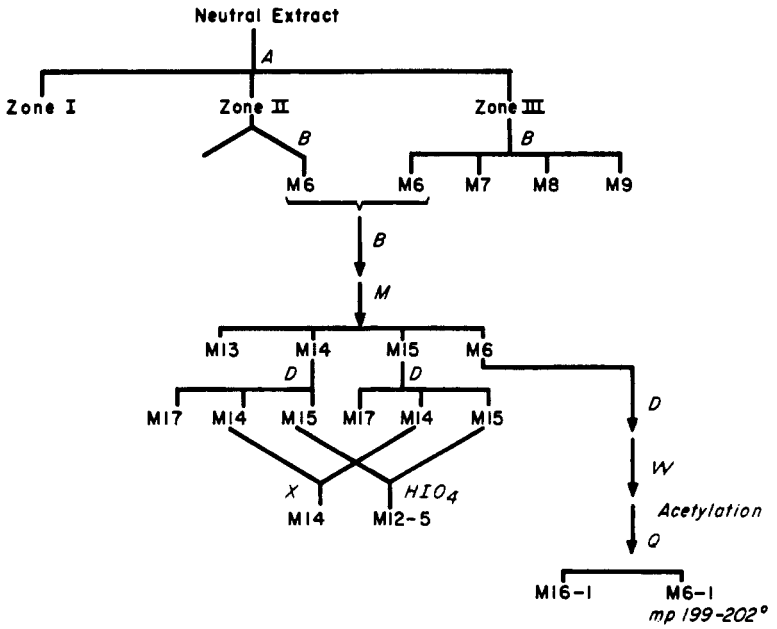

FIG. 1.—Flow sheet for the isolation of the aldosterone metabolites described in this paper. The letters A, B, D, M, Q, W, and X refer to the partition systems employed in the column chromatography as given in Table I. Zones I, II, and III were described previously (Kelly *et al.*, 1962a). M6 through M17 designate metabolites of aldosterone as described in the text, and M16-1, M6-1, and M12-5 are derivatives of the metabolites also as described in the text.

radioactive substances were eluted from the last two chromatograms.

Acetylation of M6; M6-1 and M16-1.—In spite of the repeated chromatography urinary pigments were still present in sufficient amount to prevent the determination of the infrared spectrum of M6, although 235,000 cpm, equivalent to about 2.3 mg, were present in the sample (the specific activity of the metabolites isolated in this study was about 100,000 cpm/mg). The metabolite was acetylated with C^{14}-acetic anhydride of known specific activity in pyridine at room temperature for 18 hours. The reaction mixture was diluted with 5 ml of benzene and then evaporated to dryness. Upon chromatography on system Q, the residue gave rise to a monoacetate, M16-1, eluted just behind the solvent front, and a diacetate, M6-1, eluted in 3.0 hold-back volumes. The number of acetate groups in each derivative were determined as previously described (Kelly *et al.*, 1962a,b). The pertinent data are given in Table II.

M6-1 yielded about 0.5 mg of crystals, mp 199–202°, from acetone-heptane. The infrared spectrum (Figure 4) displayed bands characteristic of a C_{18}-hemiacetal acetate (1760, 1220 cm^{-1}, Kelly *et al.*, 1962b), acetate (1735, 1240 cm^{-1}), and a ketone (1710 cm^{-1}).

M16-1 did not crystallize, and its infrared spectrum

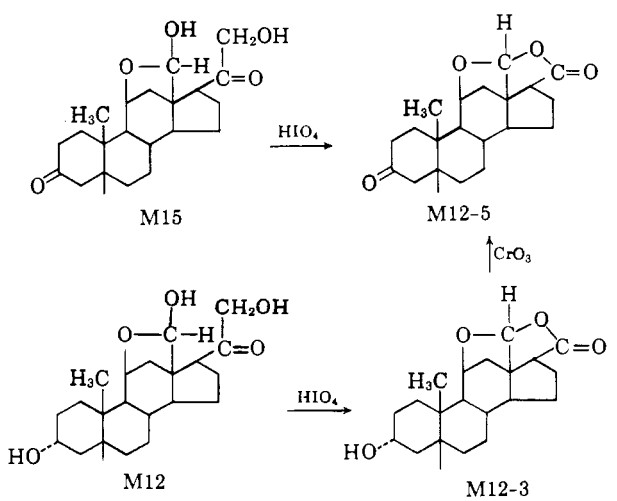

FIG. 2.—The characterization of M15 by conversion to M12-5 whose partial synthesis from M12 was previously described (Kelly *et al.*, 1962b).

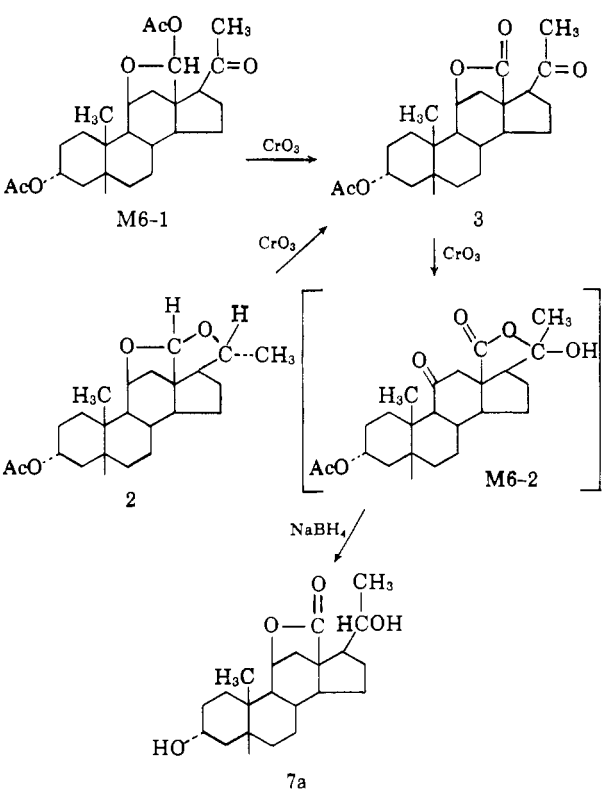

FIG. 3.—The characterization of M6.

TABLE I

PARTITION SYSTEMS EMPLOYED IN COLUMN CHROMATOGRAPHY

System	Components	Celite (g/ml 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
D	Isooctane 0.5, *t*-butanol 0.25, water 0.45, methanol 0.1	2
I	Ethyl acetate 0.33, hexane 0.67, methanol 0.35, water 0.15	2
M	Methylene chloride 0.5, methyl cyclohexane 0.5, ethylene glycol 0.1	2
Q	Heptane 1, methanol 0.9, water 0.1	2
U	Benzene 1, ligroin 3, methanol 2, water 1	2
W	Ligroin 1.2, benzene 2.8, methanol 2.0, water 1.0	2
X	Ligroin 2, benzene 2, methanol 2, water 1	2
E_4	Isooctane 0.5, *t*-butanol 0.225, water 0.05, methanol 0.225[a]	2

[a] Eberlein and Bongiovanni (1955).

TABLE II

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

Acetate	H^3/C^{14}	Number of Acetylatable Hydroxyl Groups
M16-1	17.4	1.1
M14-1	9.0	2.1
M6-1	9.2	2.1

[a] Specific activity of the H^3 metabolites = 44,000 cpm/μmole. Specific activity of the C^{14}–acetic anhydride = 2300 cpm/μeq. Counting conditions and calculations as described previously (Kelly *et al.*, 1962a). All counting data are corrected for fluctuations in counting efficiency.

(Figure 4) indicated the presence of the acetoxy group (1735 cm^{-1} and 1240 cm^{-1}). The band at 1710 cm^{-1} does not seem to be strong enough to indicate the presence of a ketone in the metabolite; rather it appears to arise from an impurity in the sample. Because of the limited quantity available, M16-1 was not further investigated.

Separation of M14 and M15.—The poorly separated M14 and M15 from the chromatogram in system M were separately chromatographed in system D. In each case, three radioactive substances were eluted at 1.4, 2.2, and 5.6 hold-back volumes, respectively, and these were designated M17, M14, and M15, respectively. The minute quantity of M17 present precluded further investigation.

M15.—The fractions containing M15 were combined and an aliquot was treated with the blue tetrazolium reagent (Recknagle and Littera, 1956). In this way about 420 μg of this metabolite was estimated to be present. Because of contamination of the sample with urinary pigment, the infrared spectrum could not be determined.

M14.—The fractions eluted at 2.2 hold-back volumes from the chromatogram in system D were combined and rechromatographed in system X. Most of the radioactivity was eluted in 7.2 hold-back volumes. The total radioactivity comprising this peak was

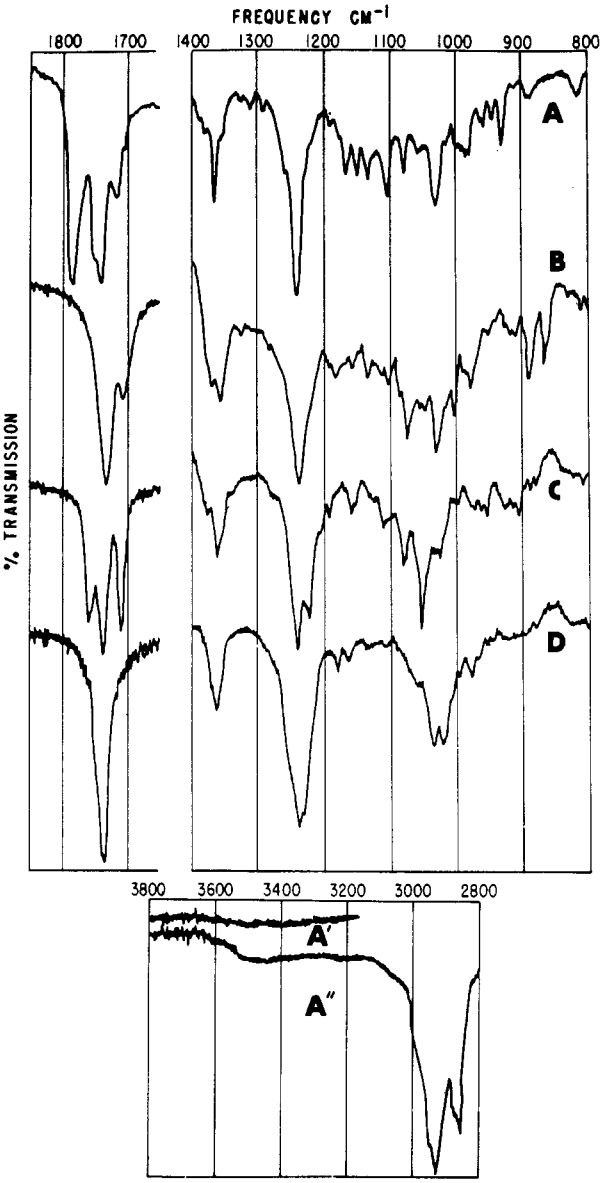

FIG. 4.—Infrared spectra of acetoxy derivatives of metabolites of aldosterone in CS_2 solution.

A M6-2
A′ CS_2 blank in the sample cell
A″ M6-2
B M16-1
C M6-1
D M14-1

about 40,000 cpm, which was equivalent to about 0.4 mg of the metabolite. However, the sample was still not sufficiently pure for infrared analysis.

M14-1.—The entire sample of M14 was acetylated with C^{14}-labeled acetic anhydride in pyridine at room temperature for 18 hours. Five ml of benzene was then added to the reaction mixture, and the resulting solution was evaporated to dryness under a stream of nitrogen. The residue was chromatographed in system Q and the radioactivity was eluted as an oil in 2.2 hold-back volumes. Determination of H^3/C^{14} ratio (see Table II) established that M14-1 was a diacetate. M14-1 absorbed infrared radiation at 1735 and 1240 cm^{-1} (acetate) and at 1020 cm^{-1} and 1030 cm^{-1}. The spectrum is presented in Figure 4.

Structural Studies

M15 (Figure 2). Oxidation to M12-5.—To a solution

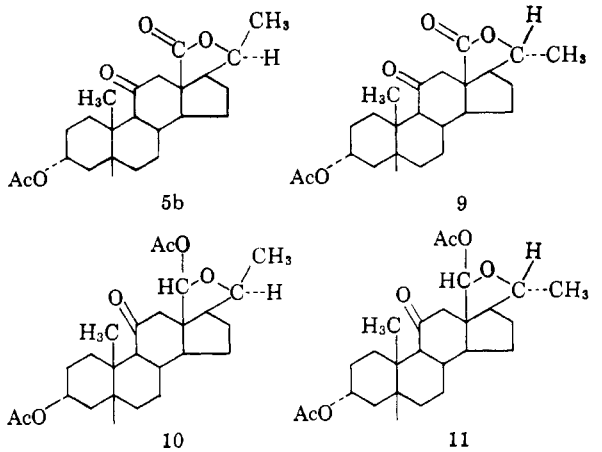

FIG. 5.—Possible structures for M16-1 and the corresponding ketolactone acetates.

of crude M15 in 0.15 ml of methanol was added 0.8 ml of a solution of 225 mg of $HIO_4 \cdot 2\ H_2O$ and 0.1 ml pyridine in 10 ml of water. The reaction mixture was allowed to stand for 18 hours at room temperature. It was then poured into 100 ml of water, which was extracted three times with 100 ml of methylene chloride. The combined methylene chloride extracts were washed first with $NaHCO_3$ solution and then with water, dried over Na_2SO_4, and evaporated to dryness *in vacuo*. The dry residue was chromatographed in system U. A crystalline ketolactone, identified by infrared spectroscopy as M12-5 (11β-hydroxy-18-oxo-3-keto-5β-etianic acid (18 → 11) hemiacetal (20 → 18) lactone) (Kelly *et al.*, 1962b; Ulick and Vetter, 1962) was eluted in 2.0 hold-back volumes. The partial synthesis of M12-5 from M-12 is shown in Figure 2.

M6 (Figure 3). Oxidation of M6-1 to M6-2.—The 0.5 mg sample of crystalline M6-1 was treated with 0.2 ml of 2% CrO_3 in 90% acetic acid for 18 hours. The reaction mixture was poured into 100 ml of water, and the mixture was extracted three times with methylene chloride. The combined organic extracts were washed first with $NaHCO_3$ and then with water, were dried over Na_2SO_4, and evaporated to dryness *in vacuo*. Upon chromatography of the residue on the E_4 system, a lactol formulated as 3α-acetoxy-11,20-diketopregnan-18-oic acid (18 → 20) lactol (M6-2) was eluted in 2.6 hold-back volumes. The infrared spectrum of this substance, shown in Figure 4, displayed bands characteristic of lactone or lactol (1780 cm^{-1}), acetate (1735, 1240 cm^{-1}), and ketone (1710 cm^{-1}). In addition the presence in the spectrum of a broad (3600 cm^{-1} to 3000 cm^{-1}) weak band indicative of a strongly hydrogen-bonded hydroxyl group is apparent.

Synthesis of 3α-Acetoxy-11,20-diketopregnan-18-oic Acid (18 → 20) Lactol, M6-2, from Ketolactone Acetate (3)

One mg of 3α-acetoxy-11β-hydroxy-20-ketopregnan-18-oic acid (18 → 11) lactone (3)[1] was dissolved in 0.5 ml of 2% CrO_3 in 90% acetic acid. After 2.5 hours at room temperature, the reaction mixture had become dark brown. At this time it was poured into 100 ml of water, which was then extracted with methylene chloride. The methylene chloride extract was washed first with $NaHCO_3$ solution and then with water, dried over Na_2SO_4, and evaporated to dryness under vacuum. The infrared spectrum of the oily residue M6-2 was identical with that of the chromic acid oxidation product of M6-1 and with that of the oxidation product of 3α-acetoxypregnane (11β → 18S)(18S-20β) dioxide (2) (Kelly *et al.*, 1963).

Reduction of M6-2 to Dihydroxylactone (7a) (Figure 3).—To a solution of 1 mg of M6-2 in 1 ml of ethanol and 1 ml of tetrahydrofuran was added a solution of 10 mg of $NaBH_4$ in 1 ml of water. This reaction mixture was allowed to stand at room temperature for 3 days. The mixture was then poured into 100 ml of water, acidified with acetic acid, and extracted three times with methylene chloride. The combined extracts were washed with water until free of acid, dried over Na_2SO_4, and, finally, evaporated to dryness under vacuum. Upon chromatography of the residue in system I, a crystalline substance identified as (7a) (Kelly *et al.*, 1963) by infrared spectroscopy was eluted in the fifth hold-back volume.

Attempted Acetylation of M6-2 with Acetic Anhydride.—One mg of M6-2 was treated for 18 hours at room temperature with 0.1 ml of pyridine and 0.05 ml acetic anhydride. To the reaction mixture was added 5 ml of benzene, and the whole was evaporated to dryness under a stream of nitrogen. The residue was identified by infrared spectroscopy as unchanged M6-2.

Treatment of (5b) with CrO_3 (Figure 5).—One mg of 3α-acetoxy-20α-hydroxy-11-ketopregnan-18-oic acid (18 → 20) lactone (5b) (Kelly *et al.*, 1963) was treated with 0.5 ml of 2% CrO_3 in 90% acetic acid. After 18 hours at room temperature, the reaction mixture had not changed color. The product was isolated as described above and identified by infrared spectroscopy as the unchanged starting material.

Treatment of (9) with CrO_3 (Figure 5).—One mg of 3α-acetoxy-20β-hydroxy-11-ketopregnan-18-oic acid (18 → 20) lactone (9) (the preparation of this compound from 20β-hydroxy-11α-acetoxy-3-ketopregnan-18-oic acid (18 → 20) lactone[1] will be described in a subsequent publication) was treated as above. No reaction occurred as evidenced by the recovery of the starting material.

RESULTS AND DISCUSSION

Structure of M15.—The chromatographic mobility of M15 and its reaction with blue tetrazolium suggested that this metabolite was one of the two possible 4,5-dihydroaldosterones. M15 could not be isolated in a form pure enough for characterization as the metabolite. However, upon oxidation with periodic acid, it gave the same crystalline ketoetiolactone as previously obtained from tetrahydroaldosterone (M12) (Kelly *et al.*, 1962b; Ulick and Vetter, 1962) (see Figure 2). Hence the conversion of M15 to M12-5 established the structure of M15 as 11β-21-dihydroxy-18-oxopregnane-3,20-dione. Although the losses incurred during the isolation of this metabolite were large, it would seem unlikely that more than 1–2 mg was originally present in the urine. This represents less than 1% of the 760 mg of aldosterone administered in this study.

Structure of M6.—Although pure M6 could not be isolated, even after repeated chromatography, its diacetate, M6-1, was obtained in crystalline form. The presence of strong bands at 1760 cm^{-1} and 1220 cm^{-1} in the infrared spectrum of M6-1 suggests that one of the two acetates is an ester of a hemiacetal. Similar bands have been observed in the spectrum of the 18-monoacetate-3,21-dibenzoate of tetrahydroaldosterone (Kelly *et al.*, 1962b). Furthermore, the infrared spectrum also indicated the presence in M6-1 of a ketone group (1710 cm^{-1}) which could be located at C_3, C_{11}, or C_{20}. The oxidation by chromic acid of M6-1 to the lactol, 3α-acetoxy-11,20-diketopregnan-18-

[1] Generously provided by Dr. A. Wettstein, Ciba Ltd., Basle, Switzerland.

oic (18 → 20) lactol, M6-2, established that the acetoxy group was at C_3 and that C_{21} was a methyl group. From the evidence present thus far, three isomeric structures (10, 11, and M6-1) could be proposed for M6-1, as shown in Figures 3 and 5. These differ in the position of the ketone; if the ketone were at C_{11}, then two compounds epimeric at C_{20} are possible, namely, (10) and (11) (Figure 5).

The oxidation of each of the three proposed structures would be expected to afford the corresponding keto-lactone acetate (3), (5b), and (9) (Figures 3 and 5) rather than the lactol M6-2 which was actually obtained. However, the lactol might be regarded as a further oxidation product of one or more of the three expected lactones. When this hypothesis was tested by treating each of the three lactones (3, 5b, and 9) with excess chromic acid in acetic acid, the (18 → 11) lactone (3) was converted to M6-2 in 2.5 hours (Figure 3), whereas the (18 → 20) lactones (5b) and (9) were recovered unchanged after 18 hours. Moreover, experiments of Heusler *et al.* (1961) imply that (18 → 20α) lactones are stable to CrO_3 in acetic acid. Therefore the diacetate of the metabolite cannot have either structure (10) or (11), as these would be oxidized to (5b) or (9), respectively, but not to M6-2. On the other hand, since (3) did give rise to M6-2, the diacetate M6-1 must have the structure shown in Figure 3. The lactol M6-2 was characterized further by reducing it to the known (Kelly *et al.*, 1963) 3α,20α-dihydroxy lactone (7a) with $NaBH_4$. Moreover, although the infrared spectrum of M6-2 demonstrates that the lactol possesses a hydroxyl group, treatment of it with acetic anhydride in pyridine failed to produce the corresponding acetate. Thus the structure shown in Figure 3 is tentatively suggested for M6-2. Synthetic steroid C_{18} lactols have been described by Velluz *et al.* (1960), by Lábler and Šorm (1959a,b), and by Cainelli *et al.* (1959).

The isolation of M6 was difficult and the losses were probably quite large. At one point in the procedure, about 550,000 cpm representing about 5.5 mg were recorded. Probably somewhat more than this was actually present in the urine, representing perhaps about 1% of the administered aldosterone. M6 is the second 21-deoxy metabolite of aldosterone to be isolated from human urine. The metabolic significance of the 21-dehydroxylation of aldosterone in man has already been discussed (Kelly *et al.*, 1962a). However, the previously described urinary 21-deoxy metabolites of C_{21} hydroxy corticoids are the corresponding C_{20} alcohols. M6 is the first urinary 20-keto product derived from a corticosteroid by 21-dehydroxylation to be recognized as such.

The structure of M6 suggests that it may be a precursor of the bicyclic acetal M1, which is one of the major urinary metabolites of aldosterone (Kelly *et al.*, 1962a) (see Figure 6). This would imply that the C_{21} hydroxyl group is removed prior to the reduction of the 20-ketone and prior to the subsequent bicyclic acetal formation. A second possible pathway of formation of M1, illustrated in Figure 6, could be via 21-dehydroxylation of M8, a 3α,21-dihydroxy bicyclic acetal isolated from human urine in somewhat larger amounts than M6 (Kelly *et al.*, 1962a).

M13, M14, M16, and M17.—The isolation of a number of metabolites of aldosterone in amounts too small to permit characterization has been reported (Kelly *et al.*, 1962a). In this study several previously unknown metabolites were isolated, in some cases in amounts large enough to permit partial characterization. It is possible that M13 and M17 may be the same substance, and that this may also be the same as the previously reported M7 (Kelly *et al.*, 1962b). M16 was separated from M6 only after acetylation. Since M16-1 was a monoacetate which was eluted from system Q at the solvent front, it may be an artifact of acetylation either of M6 or even of another metabolite. No additional information regarding its structure or its origin could be obtained from its infrared spectra.

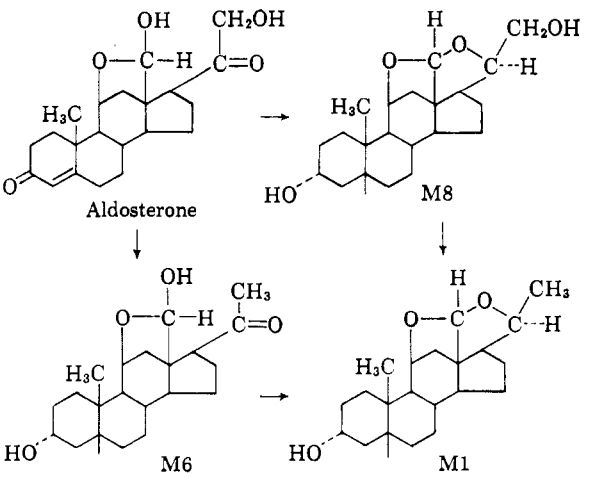

FIG. 6.—Hypothetical precursors for the 21-deoxy bicyclic acetal metabolite, M1.

It was not possible to isolate pure M14. However, acetylation of the crude metabolite afforded the diacetate M14-1, whose infrared spectrum did not display a band between 1720 cm^{-1} and 1650 cm^{-1}, indicating that the metabolite M14 contained no carbonyl group. A pair of strong bands suggestive of the presence in M14-1 of a bicyclic acetal were observed in the 1000–1050 cm^{-1} region. However, comparison of M14-1 with M8 diacetate (Kelly *et al.*, 1962a) (the structure of M8 is shown in Figure 6) established that the two were not the same. The absence of a strong band near 1075 cm^{-1} in the spectrum of M14-1 suggests that M14-1 is probably not the 20β epimer of M8 acetate (the infrared spectra of bicyclic acetals has been discussed by Kelly *et al.*, 1962a, 1963). The smooth contour of the band near 1240 cm^{-1} indicates that if a 3-acetoxy group is present it is equatorial. The absence of bands near 1760 cm^{-1} and 1225 cm^{-1} render it unlikely that M14-1 contains a hemiacetal acetate.

Chromic Acid Oxidation of (18 → 11β) Lactones.—As previously pointed out (Kelly *et al.*, 1962a, 1963) the oxidation of M1 monoacetate with CrO_3 in acetic acid gave the ketolactone acetate (3), whereas similar treatment of the synthetic 20β epimer of M1 acetate gave (3) and M6-2 together with unreacted bicyclic acetal. The difference in rate of oxidation was ascribed to the different steric effects of the C_{21} methyl groups. The formation of M6-2 can now be explained as due to the oxidation of (3) by excess CrO_3 in the reaction mixture. The amount of M6-2 formed could probably be minimized by the slow addition of CrO_3 to the reaction mixture so that the excess of reagent present at any time would be slight. The rate of oxidation of M1 acetate, on the other hand, appears to be greater than the rate of oxidation of (3), with the result that when an excess of reagent is added at the beginning of the reaction (3) is still the only oxidation product formed. Although not anticipated, the oxidation of (18 → 11β) lactones to 11-keto-18-oic acids is in keeping with the well-established rapid rate of oxidation of 11β alcohols to 11-ketones. Conversely, the failure of (18 → 20) lactones to be oxidized under similar conditions reflects the relatively slower rate of

oxidation of C_{20} alcohols as compared with that of 11β-hydroxyl groups.

References

Cainelli, G., Mihailovic, M. L., Arigoni, D., Jeger, O. (1959), *Helv. Chim. Acta 42*, 1124.

Eberlein, W. R., and Bongiovanni, A. M. (1955), *Arch. Biochem. Biophys. 59*, 90.

Heusler, K., Kalvoda, J., Meystre, C., Wieland, P., Anner, G., Wettstein, A., Cainelli, G., Arigoni, D., and Jeger, O. (1961), *Helv. Chim. Acta 44*, 502.

Kelly, W. G., Bandi, L., and Lieberman, S. (1962b), *Biochemistry 1*, 792.

Kelly, W. G., Bandi, L., and Lieberman, S. (1963), *Biochemistry 2*, 1249.

Kelly, W. G., Bandi, L., Shoolery, J., and Lieberman, S. (1962a), *Biochemistry 1*, 172.

Lábler, L., and Šorm, F. (1959a), *Collection Czech Chem. Commun. 24*, 2975.

Lábler, L., and Šorm, F. (1959b), *Collection Czech Chem. Commun. 24*, 4010.

Okita, G. J., Kabara, J. J., Richardson, F., and LeRoy, G. V. (1957), *Nucleonics 15*, 111.

Recknagel, R. O., and Littera, M. (1956), *J. Lab. Clin. Med. 48*, 463.

Ulick, S., and Vetter, K. K. (1962), *J. Biol. Chem. 237*, 3364.

Velluz, L., Muller, G., Bardoneschi, R., Poittevin, A. (1960), *Compt. Rend. 250*, 725.

The Chemical Synthesis of Mevalonic Acid 5-Phosphate, Isopentenyl Pyrophosphate, and Related Compounds*

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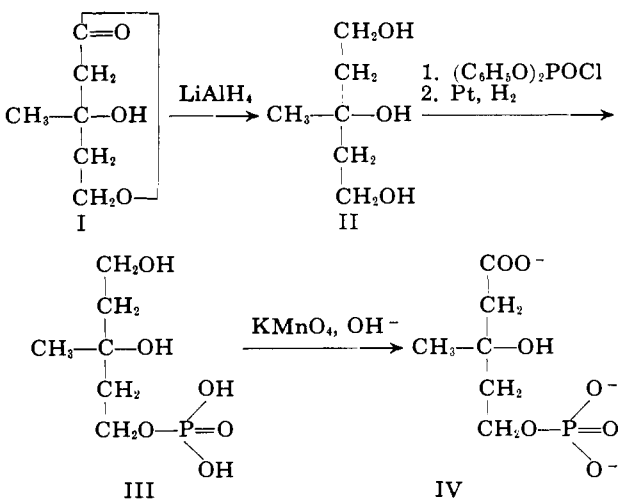
DL-Mevalonic acid 5-phosphate has been prepared by reduction of mevalonic lactone to 3-methlyl-1,3,5-pentanetriol and phosphorylation of this compound to 3-methyl-1,3,5-pentanetriol 5-phosphate, followed by oxidation to the desired product. 3-Methyl-1,5-pentanediol-5-phosphate and 5-hydroxy-3-methylpentanoic acid 5-phosphate have been prepared in a similar manner. The anhydride-anion exchange procedure for the synthesis of phosphate anhydrides has been successfully applied to the preparation of isopentenyl pyrophosphate.

Mevalonic acid 5-phosphate is well established as an intermediate in the enzymatic conversion of mevalonic acid to isopentenyl pyrophosphate (e.g., see the review by Popjak and Cornforth, 1960), but no satisfactory chemical synthesis of the compound is available (Lynen, 1959; Hellig and Popjak, 1961). In this report we describe a series of reactions which leads to mevalonic acid 5-phosphate in good yield.



Mevalonic lactone (I) was reduced to the corresponding triol (II) with lithium aluminum hydride. The phosphorylation of (II) to give the monophosphate ester (III) in reasonable yield without blocking the other alcohol functions was accomplished by reacting 1 mole of triol with 0.5–0.7 mole of diphenyl phosphorochloridate at room temperature. Under these conditions tertiary alcohols do not react (Wold and Ballou, 1959) and the ratio of monoester to diester produced was quite favorable. The monoester was separated from the diester by ion-exchange chromatography, and in the same step the unreacted triol was readily recovered. The over-all yield based on consumption of triol was 60–70%. Attempts to prepare the monoester after first blocking two of the three alcohol functions by preparing either the isopropylidene or ethylidene derivatives of the triol did not result in any improvement of this yield.

Oxidation of 3-methyl-1,3,5-pentanetriol 5-phosphate (III) with an excess of alkaline permanganate (Ballou and Hesse, 1956) yielded a compound which analyzed correctly for mevalonic acid 5-phosphate (IV). Potentiometric titration of this compound demonstrated that it contained one carboxyl group (pK about 4) per mole of phosphorus. The only structure for this final product which is consistent with these results is that of mevalonic acid 5-phosphate (IV). The over-all yield from mevalonic lactone was 43%.

A crude enzyme extract prepared from rabbit liver was used to prepare mevalonic acid 5-phosphate from 2-C^{14}-mevalonic lactone and ATP. 2-C^{14}-mevalonic acid 5-phosphate was separated from 2-C^{14}-mevalonic acid 5-pyrophosphate and isopentenyl pyrophosphate by paper chromatography (Tchen, 1958; Witting and Porter, 1959), and after it was eluted from the paper the enzymatically prepared 2-C^{14} compound was mixed with the synthetic product and chromatographed on an ion-exchange column. Figure 1 shows the exact match-

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