Isolation, Determination of Structure and Synthesis of the Acid-Labile Conjugate of Aldosterone

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1. After administration of 600 mg of ^3H -labelled aldosterone to human volunteers, 57 mg of homogeneous acid-labile conjugate was isolated from the urine and identified as aldosterone 18 β -D-glucosiduronic acid. 2. Esterification and acetylation of the conjugate gave a tetra-acetate methyl ester, which, by measurement of the optical rotation and nuclear-magnetic-resonance spectrum, was shown to be a β -glucosiduronate. This tetra-acetate methyl ester was synthesized in approx. 10% yield by the Koenigs-Knorr procedure. 3. Removal of the acetyl and methyl ester groups from the tetra-acetate methyl ester with alkali was accompanied by almost complete isomerization at C-17 to give 17-isoaldosterone 18 β -D-glucosiduronic acid. 4. To prevent inversion at C-17 during removal of the acetate and ester groups of β -glucosiduronate (a) the 3,20-disemicarbazone was prepared, (b) the acetate and ester groups were removed from the disemicarbazone by treatment with alkali, and (c) the semicarbazone groups were removed from the product at pH 2.0, and aldosterone 18 β -D-glucosiduronic acid was obtained in 47% overall yield. 5. In the presence of components used to synthesize β -glucosiduronate by the Koenigs-Knorr reaction this substance is converted slowly into the α -glucosiduronate; this conversion is responsible, in part, for the low yield of β -glucosiduronate. 6. Two additional conjugates were obtained in the Koenigs-Knorr reaction; a provisional structure was assigned to one substance. The other substance is a C-18 α -glucosiduronate. Removal of the acetyl and ester groups from C-18 α -glucosiduronate gave the α -glucosiduronic acid in 84% yield and the 17-isoaldosterone α -glucosiduronic acid in 12% yield. 7. The rate at which several types of β -glucuronidase hydrolyse the foregoing steroidal α - and β -glucosiduronic acids is given.

Aldosterone occurs in human urine in part as a highly polar conjugate, which can be hydrolysed at pH 1.0 to give aldosterone. This metabolite has been referred to as the 3-oxo conjugate, the pH 1.0 conjugate and the acid-labile conjugate. After administration of [^3H]aldosterone to human volunteers, the conjugate was isolated from urine by Underwood & Tait (1964) and Pasqualini *et al.* (1965), and formulated provisionally as aldosterone 18-glucosiduronic acid.* The product is reported to be unique in that it is a steroidal glucosiduronate that is resistant to hydrolysis by β -glucuronidase (Ketodase) and that it is hydrolysed readily by dilute mineral acids, whereas conventional steroidal glucosiduronates are hydrolysed very slowly by weak mineral acids. The conjugate has occupied an important position in clinical chemistry; its hydrolysis has yielded aldosterone which, when quantified, has served to detect various states of hypoproduction and hyperproduction of this hormone.

* Simplified names are used in this introduction, the Results and Discussion section and Tables, and systematic nomenclature in the Experimental section.

Underwood & Frye (1972) attempted to synthesize the polyacetate methyl ester of the conjugate by employing aldosterone 21-acetate and methyl acetobromoglucuronate in the Koenigs-Knorr reaction. They obtained small amounts of two substances which, from their mass spectra, i.r. spectra and chemical properties, were thought to be isomeric C-18 methyl triacetylglucosiduronates of aldosterone 21-acetate. However, the relationship of these compounds to the acid-labile conjugate was not determined.

Our interest in steroidal glucosiduronates (Mattox *et al.*, 1969; Mattox & Vrieze, 1972) has impelled us to attempt to establish the structure of the acid-labile conjugate of aldosterone. We chose, initially, to attempt to synthesize the two substances (P_1 and P_2) prepared by Underwood & Frye (1972) and to establish the structures of these compounds.

Results and Discussion

Treatment of aldosterone 21-acetate (I) with methyl acetobromoglucuronate and Ag_2CO_3 followed by chromatographic resolution gave two products.

Scheme 1.

One substance (m.p. 135–139°C) is formulated tentatively as compound (II) (Scheme 1). This structure represents a glucuronate rather than a glucosiduronate. The formulation is based on the following evidence. Results of the elemental analysis are consistent with the structure suggested. The presence of the 3-oxo- Δ^4 function is shown by the u.v. and i.r. spectra; the presence of the ketol side chain is suggested by a positive Tetrazolium Blue reaction and confirmed by the n.m.r. spectrum (Table 1). Compound (II), upon treatment with semicarbazide, forms a disemicarbazone (III). Treatment of compound (II) with hydroxylamine by the method of Caplow & Jenks (1964) demonstrated the presence of five ester groups in the

compound. The foregoing results imply that the glucuronyl moiety is not attached to the steroid at C-21, C-20 or C-3; it seems probable that it is attached at C-18. The substance is not an orthoacetate, because it does not undergo the facile hydrolysis of this type of compound (Mattox & Vrieze, 1972) and its n.m.r. spectrum lacks the proton band that is characteristic of orthoacetates; in addition, no signal for $\text{CH}_3\text{C}(\text{OR})_3$ was observed in the ^{13}C n.m.r. spectrum. Treatment of compound (II) with dilute methanolic NaOH gave atypical results in that a strong yellow colour developed and, as judged by t.l.c., multiple products were formed rather than a principal substance and traces of impurities. Consideration of the ^1H n.m.r. spectrum of compound (II)

Table 1. *N.m.r. spectra of conjugates of aldosterone*

Coupling constants of approx. 7.5Hz and approx. 3.5Hz for C-1' H are characteristic of β - and α -glycosidic linkages respectively. The sample of compound (VIII) that was derived from the natural acid-labile conjugate, is identical with the sample of compound (VIII) that was prepared by chemical synthesis. Nature of signal: s, singlet; d, doublet; dd, double doublet; t, triplet. Coupling constants (J_{xy}) are in parentheses.

Proton(s)	Compound	Chemical shift (δ) (p.p.m.)			
		... (II)*	(IV)*	(VIII)† (natural)	(VIII)† (synthetic)
C-19 H ₃	s	1.26	1.21	1.25	1.25
	s	2.03	2.00	2.01	2.00
O	s	2.08	2.06	2.02	2.02
	s	2.13	2.11	2.08	2.09
-C-CH ₃	s	2.35	2.18	2.15	2.15
C-17 H	t	2.82	3.07	2.70	2.70
CO ₂ CH ₃	s	3.72	3.76	3.74	3.74
C-5' H	d	4.31 (9.7)	4.21 (9.5)	4.06 (9.4)	4.06 (9.4)
C-11 H	d	4.46 (6.3)	4.47 (6.3)	4.53 (6.5)	4.54 (6.4)
C-1' H	d	6.29 (3.3)	5.50 (4.3)	4.77 (7.5)	4.77 (7.5)
C-2' H	dd	4.08	4.92		
C-3' H	t	5.28 (9.5)	5.26 (9.2)		
C-4' H	t	5.17 (9.5)	5.16 (9.2)		
C-21 H	{ d d	4.46 4.52 (15.5)	4.73 4.99 (16.0)	} 4.77 s	4.77 s
C-18 H	s	4.68	4.94		
C-4 H	s	5.70	5.70	5.70	5.70

* See Scheme 1.

† See Scheme 3.

(Table 1) in relation to the spectra of compounds (IV) and (VIII) suggests that the three compounds have an identical steroid moiety and that these substances are isomeric in the glucuronate portion of the molecule. In compound (II), the signal for the C-1' proton ($\delta = 6.29$; $J_{1',2'} = 3.3$ Hz) implies that the anomeric oxygen has the α -configuration. Comparison of the ^1H n.m.r. spectrum of compound (II) with that of compound (IV), the structure of which is firmly established, suggests that the compounds are identical at all positions except C-1' and C-2'. If it is assumed that compound (II) differs from compound (IV) solely by an interchange of the steroidal and acetoxy groups at C-1' and C-2', it follows that structure (II) (Scheme 1) should be considered for the compound of m.p. 135–139°C. This structure provides an acceptable explanation for the ^1H n.m.r. spectrum and general properties of compound (II). Comparison of the data in the paper by Underwood & Frye (1972) with that for the foregoing compound suggests that compound (II) and P₁ are identical.

All of the natural steroidal glucosiduronates that have been characterized have been β -glycosidic substances. When it became apparent that the foregoing compound (II) was not a typical β -glucosiduronate and thus probably was not the polyacetate ester of the acid-labile conjugate, attempts to estab-

lish an unequivocal structure for the substance were discontinued.

A second substance formed in the Koenigs–Knorr reaction is compound (IV), which is identical with substance P₂ of Underwood & Frye (1972). The molecular rotation (Table 2) indicates that the substance is an α -glucosiduronate and the n.m.r. spectrum (Table 1; signal at $\delta = 5.50$, anomeric H, $J_{1',2'} = 4.3$ Hz) confirms this configuration. An analysis of the X-ray diffraction pattern of (IV) (W. L. Duax & C. M. Weeks, personal communication) verifies the presence of an α -glycosidic linkage in the compound and, in addition, reveals that the configuration at C-18 is *R*. From this latter finding it follows that compounds (V), (VI) and (VII), which are derived from compound (IV), also have the *R* configuration at C-18.

In the presence of alkali, ($\pm$)-aldosterone tends to undergo inversion at C-17 and form 17-iso-($\pm$)-aldosterone (Schmidlin *et al.*, 1957); inversion at C-17 is prone to occur also during treatment of polyacetate esters of (+)-aldosterone 18-glucosiduronic acid with alkali. Transesterification of the tetra-acetate (IV) in dilute methanolic alkali gave the ester (V) as an intermediate, which, upon treatment with dilute aq. alkali, gave a mixture of the α -glucosiduronic acid (VI) and its 17-iso derivative (VII); these acids were readily separable by column

Table 2. *Molecular optical rotations of anomeric aldosterone glucosiduronates*

Differences in molecular rotation (ΔM_D) of α - and β -glucosiduronates of aldosterone are of the same sign and of similar magnitude as differences found for previously prepared anomeric pairs of steroidal glucosiduronates. $M_D = [\alpha]_D \times \text{mol. wt.} / 100$.

Compound number	Derivative of aldosterone 18-glucosiduronate	Configuration at C-1'	M_D	ΔM_D
IV	Tetra-acetate ester	α	+1652°	+829°
VIII	Tetra-acetate ester	β	+ 823°	
VI	Free acid	α	+1476°	+660°
XII	Free acid (synthetic)	β	+ 815°	
XII	Free acid (natural)	β	+ 816°	
20 anomeric pairs (Schneider, 1970) of steroidal glucosiduronates				+823°

chromatography. Hydrolysis of compound (VI) at pH1.0 gave aldosterone; hydrolysis of compound (VII) gave 17-iso-aldosterone. Esterification of the α -glucosiduronic acid (VI) with diazomethane gave the methyl ester (V), which, on acetylation, gave the tetra-acetate ester (IV).

After we had established that compound (VI) was aldosterone 18 α -D-glucopyranosiduronic acid, it seemed that thorough chromatographic evaluation of this substance would be of help in selecting chromatography systems that would be favourable for use in isolating the acid-labile conjugate from urine. To this end, the chromatographic mobility of compound (VI) was determined in numerous solvent systems (Mattox *et al.*, 1975b; P. C. Carpenter & V. R. Mattox, unpublished work) along with a group of synthetic steroidal glucosiduronates, which very likely are also present in human urine. It was assumed that if α -glucosiduronic acid (VI) was identical with the acid-labile conjugate of urine the chromatographic findings would be directly applicable to recovery of the conjugate; if the acid-labile conjugate was the β -anomer of compound (VI), or of opposite configuration at C-18, the properties of compound (VI) and the acid-labile conjugate might be sufficiently similar so that the data would be useful in selecting appropriate solvent systems. The results of the chromatographic studies suggested that use of chromatography systems E and P (see the Experimental section) in succession should provide superior separation of compound (VI) from other steroidal glucosiduronates that are normally present in human urine.

When 7.0mg of α -glucosiduronic acid (VI) was added to 1500ml of urine and the material was processed on (1) Amberlite XAD-2, (2) DEAE-Sephadex, (3) Amberlite XAD-2, (4) System E, (5) System P and (6) Amberlite XAD-2, crystalline α -glucosiduronic acid (VI) was isolated in 75% yield.

Isolation of the acid-labile conjugate from urine

After the administration of a total of 600mg of aldosterone to four normal individuals, and a small amount of [^3H]aldosterone to one of the individuals, the urine was collected, pooled and processed as shown in Scheme 2. The Amberlite XAD-2 (Bradlow, 1968; Mattox *et al.*, 1969) served to remove inorganic salts, urea etc. from the conjugates; the DEAE-Sephadex provided separation of neutral and anionic products on the basis of pK_a values and other features (Hobkirk *et al.*, 1969; Grose *et al.*, 1973). Sequential use of chromatography systems E and P gave resolution of an acid-labile conjugate from all other steroidal glucosiduronic acids present in the mixture. The foregoing procedure provided a radioactive product that was homogeneous in solvent systems E, J, K and N, and was distinctly different in properties from the synthetic α -glucosiduronic acid (VI). The product had properties in agreement with those reported for the acid-labile conjugate of aldosterone (Underwood & Tait, 1964; Pasqualini *et al.*, 1965); it absorbed strongly at 254nm, reduced alkaline Tetrazolium Blue, gave a positive soda fluorescence test, a negative Porter-Silber test and was cleaved at pH1.0 to produce aldosterone. Its mobility relative to that of tetrahydrocortisone glucuronide in system I of Underwood & Tait (1964) and in system III of Pasqualini *et al.* (1965) corresponded to that reported by these authors.

From previous chromatographic studies it may be implied that in the presence of a liquid ion-exchanger the extent to which the mobility of a steroidal glucosiduronate changes with a change in concentration of counterion is dependent on the number of carboxyl groups present in the conjugate (Mattox *et al.*, 1975a). We utilized this technique to obtain preliminary evidence that the acid-labile conjugate (XII) actually contains one and only one carboxyl

Scheme 2. Isolation of the acid-labile conjugate of aldosterone from urine

Hatched areas represent zones that contained the aldosterone 18 β -glucosiduronic acid. See the text for details. Aldo 18- β -GA, aldosterone 18- β -glucosiduronic acid; H₄-Aldo 3- β -GA, tetrahydroaldosterone 3- β -glucosiduronic acid; GA, glucosiduronic acid; NC, neutral compounds; S, sulphates.

group. Table 3 shows that the difference in mobility (ΔR_M) of the neutral compounds in the two concentrations of potassium acetate is +0.03; ΔR_M of the dicarboxylic acids is -0.83 and ΔR_M of the monocarboxylic acids is -0.41; ΔR_M found for the acid-labile conjugate (XII) is -0.41.

A steroidal glucosiduronate should exist as the free acid, rather than the salt, if it is to be esterified quantitatively by diazomethane. A steroidal glucosiduronate can be converted into the carboxyl form and freed of salt(s) by adsorbing it on Amberlite XAD-2, washing the column with 4-6 bed vol. of

Table 3. *Chromatographic demonstration of a carboxyl group in the acid-labile conjugate of aldosterone*

In a chromatography system that contains tetraheptylammonium acetate, the change in mobility, ΔR_M , of a steroidal glucosiduronate with the change in concentration of potassium acetate in the stationary phase is proportional to the number of carboxyl groups present. $R_M = \log[(1/R_F) - 1]$. GA, D-glucopyranosiduronic acid; GMe, D-glucopyranosiduronic acid methyl ester; E, cortisone; THA, 3 α ,21-dihydroxy-5 β -pregnane-11,20-dione; Aldo, aldosterone; THQ, 3 α ,21-dihydroxy-5 β -pregnan-20-one; α and β refer to the configuration of the glycosidic linkage.

Compound	R_M		ΔR_M	Carboxyl group(s)
	Potassium acetate concn. ...			
	0.1M	0.4M		
E 21- β -GA	-0.73	-0.28	-0.45	One
THA 3- β -GA	-0.19	+0.20	-0.39	One
Aldo 18- α -GA	-0.50	-0.10	-0.40	One
Aldo 18- β -GA*	-0.43	-0.02	-0.41	One
THQ 3,21-di- β -GA	-0.04	+0.79	-0.83	Two
THA 3,21-di- β -GA	+0.37	+1.20	-0.83	Two
E 21- β -GMe	-0.18	-0.19	+0.01	None
Aldo 18- α -GMe	-0.48	-0.55	+0.07	None
Aldo 18- β -GMe	-0.10	-0.10	0.00	None

* This substance is the acid-labile conjugate that is being examined for the presence of a carboxyl group.

5mm-H₂SO₄ or 0.1M-acetic acid, with water until the effluent is neutral and then eluting the steroidal glucosiduronic acid with methanol.

The acid-labile conjugate (acid form) was esterified with diazomethane and the resulting ester was acetylated with acetic anhydride/pyridine to yield a crystalline dimorphous product of m.p. 184–187°C and 224–228°C, which was shown later to be compound (VIII). The dimorphous product (VIII) is distinctly different in properties (m.p. and i.r. spectrum) from substances P₁ and P₂ of Underwood & Frye (1972) and from compounds (II) and (VI) that we have synthesized. Assuming that the dimorphous compound is a glucosiduronate, the doublet in the ¹H n.m.r. spectrum ($\delta = 4.77$, anomeric H, $J_{1,2} = 7.5$ Hz) indicates that the glycosidic linkage has the β configuration. The ¹H n.m.r. spectrum as a whole, when considered in association with the analysis for C and H, the i.r. spectrum and various other properties suggests strongly that the substance is methyl 21-acetoxy-11 β -18-epoxy-3,20-dioxopregn-4-en-18-yl 2,3,4-tri-O-acetyl- β -D-glucopyranosiduronate (VIII).

Synthesis of the C-18 β -glucosiduronic acid of aldosterone

With the ³H-labelled methyl tetra-acetyl β -glucosiduronate (VIII) available, it was feasible to treat aldosterone 21-acetate with methyl acetobromoglucuronate and Ag₂CO₃ and subsequently add the radiolabelled β -glucosiduronate for use as a marker during chromatography to determine whether any β -glucosiduronate was formed by the Koenigs–Knorr reaction. By using this approach, the radiolabelled marker was located and synthetic β -glucosiduronate

(VIII) was obtained in a yield of approx. 10%. The peak containing β -glucosiduronate (VIII) had been disregarded in the initial Koenigs–Knorr reactions, because under the conditions used it represented less than 5% of the total product (as judged by u.v. absorbance and tetrazolium reduction) and it emerged considerably later on the chromatogram than peaks that contained compounds (II) and (IV). Since a β -glucosiduronate is normally the principal product of the Koenigs–Knorr reaction when Ag₂CO₃ is used as coupling agent, it had not seemed likely that the later-emerging substance was the desired β -glucosiduronate.

A series of more than 20 Koenigs–Knorr reactions in which the temperature, time of reaction, ratio of reactants, coupling agent (Ag₂CO₃, Ag₂O and CdCO₃), mode of dehydration (CaSO₄, azeotropic distillation), effect of light, etc. were varied gave yields of β -glucosiduronate (VIII) that ranged from almost zero to a maximum of approx. 10%. Use of an elevated temperature, a long reaction time or azeotropic distillation gave a very small yield of compound (VIII). When 50000 d.p.m. of [³H] β -glucosiduronate (VIII) was mixed with the components of the Koenigs–Knorr reaction and the mixture was stirred at room temperature (23°C) for 18h, 77% of the radioactivity was present in the α -glucosiduronate (IV) and 22% in the β -glucosiduronate (VIII). This unexpected conversion of β -glucosiduronate into α -glucosiduronate is responsible in part for the low yield of β -anomer isolated from the Koenigs–Knorr reaction.

The synthetic polyacetate ester β -glucosiduronate (VIII) gave i.r. and n.m.r. spectra identical with those of the polyacetate ester that was prepared from the

natural acid-labile conjugate. The optical rotation of synthetic compound (VIII) (Table 2), as well as its n.m.r. spectrum, indicates that the substance is a β -glucosiduronate. [It has not been possible to obtain a crystal of β -glucosiduronate (VIII) of satisfactory size and quality to establish its C-18 configuration by X-ray analysis.]

Removal of the acetate groups from compound (VIII) in methanolic NaOH (0.01M) and removal of the ester group of the product (X) with dilute aq. NaOH (0.20M) and acidification of the solution gave a very small yield of acid-labile conjugate (XII) and a much larger yield of compound (XIII); this latter

compound (XIII) has undergone inversion at C-17 to give the unnatural 17-iso derivative, which at pH1.0 undergoes hydrolysis to yield 17-isoaldosterone. The isomerization at C-17 was found to occur during the treatment of compound (VIII) with dilute methanolic NaOH (0.01M) to prepare the methyl ester; hydrolysis of the intermediate at pH1.0 yielded 17-isoaldosterone. During the treatment with alkali, the C-17 side chain of the β -glucosiduronate (VIII) undergoes inversion much more readily than does that of the α -glucosiduronate (IV). (See Schemes 1 and 3.)

To prevent inversion at C-17 in the presence of

Scheme 3.

alkali, the diketone (VIII) was converted into the disemicarbazone (IX); this product was treated with dilute methanolic alkali to remove the acetate groups and yield an intermediate (XI); compound (XI) was treated with dilute aq. alkali to remove the methyl ester group and give the disemicarbazone (XIV). At pH 2.0 the semicarbazone groups were removed from compound (XIV) and pure aldosterone 18 β -glucopyranosiduronic acid (XII), identical with the natural acid-labile conjugate, was obtained in 47% overall yield from compound (VIII).

In the preparation of the disemicarbazone (IX) from compound (VIII), reaction at C-3 to form the monosemicarbazone appears to proceed rapidly as reported previously (Mattox *et al.*, 1969), but reaction at C-20 is slow; after allowing the reaction to proceed at 50°C for 28 h, a 63% yield of disemicarbazone (IX) was obtained.

Removal of the semicarbazone groups from the intermediate (XIV) at pH 2.0 occurs rapidly and, as judged by paper chromatography of the product, less than 5% of the glycosidic linkage is hydrolysed by this treatment.

β -Glucuronidase (Ketodase) hydrolyses the C-18 β -D-glucosiduronate (XII) very slowly as compared to the C-21 β -D-glucosiduronates of cortisone and 11-dehydrocorticosterone (Table 4); this result confirms the findings of Underwood & Tait (1964) and Pasqualini *et al.* (1965) concerning hepatic β -glucuronidase. Ketodase causes almost no hydrolysis of the C-18 α -D-glucosiduronate (VI). Sigma's β -glucuronidase (type B-1; bovine liver) hydrolyses the three foregoing β -D-glucosiduronates at about the same rate and also causes significant hydrolysis of the α -D-glucosiduronate. The β -glucuronidase preparation from *Helix pomatia* hydrolyses the C-18 α -D-glucosiduronate at about the same rate as it hydrolyses the C-18 and C-21 β -D-glucosiduronates.

Experimental

General

Aldosterone 21-acetate was from Ikapharm, Ramat-Gan, Israel. [1,2,6,7-³H]Aldosterone (New England Nuclear, Boston, MA, U.S.A.) was stored in benzene/ethanol (9:1, v/v) at -5°C. Immediately before use, [³H]aldosterone was purified by column chromatography in system C using acid-washed Celite (Hyflo-Supercel). Silicone Gum Rubber SE-30 was from Analabs Inc., New Haven, CT, U.S.A. Aliquat (methyltrisioctanoylammonium chloride) was from General Mills, Minneapolis, MN, U.S.A. Solvents were of analytical-reagent grade and, with the exception of formamide, were redistilled before use. To recover reaction products, solutions were evaporated *in vacuo* at 40°C or less. Samples that were used for chemical and physical analysis had been shown to be homogeneous in two or more chromatographic systems and had been dried at

100°C immediately before being used. Melting points were determined with the Fisher-Johns apparatus and are corrected. Optical rotations were obtained at 23 $\pm$ 1°C in a 1 dm cell at $c \approx 1.0$. ϵ is expressed as litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. ¹H n.m.r. spectra were obtained in [²H]chloroform with a Varian HA-100 spectrometer (Varian Associates, Palo Alto, CA, U.S.A.) by using tetramethylsilane as the internal reference. Radioactivity was measured in Insta-Gel (Packard Instruments, Downers Grove, IL, U.S.A.) by using a Beckman LC-100 liquid-scintillation spectrometer with external standard for determining efficiencies.

Chromatography

All chromatography was performed at 23 $\pm$ 1°C. For systems lacking liquid ion exchangers, paper chromatography was performed as previously described (Mattox *et al.*, 1969). T.l.c. was carried out on unactivated plates of Silica Gel GF-254 (Applied Science, State College, PA, U.S.A.). Partition column chromatography was modified from previous descriptions (Mattox *et al.*, 1969); Celite and stationary phase were mixed in the ratio of 100 g to 50 ml. Chromatography columns were operated at a load of about 1 μ mol of steroid/ml of packed column vol.

The solvent systems were: A, benzene/cyclohexane (1:1, v/v)/formamide; B, benzene/chloroform (1:1, v/v)/formamide; C, benzene/cyclohexane/methanol/water (9:1:6:4, by vol.); D, cyclohexane/benzene/methanol/water (5:2:5:1, by vol.); E, 1,2-dichloroethane/2-methylpropan-2-ol/water/acetic acid (3:1:3:1, by vol.); F, butyl acetate/toluene/methanol/water/acetic acid (10:10:10:9:1, by vol.); G, ethyl acetate/iso-octane (2:1, v/v); H, benzene/acetone (1:1, v/v); I, ethyl acetate/methanol/acetic acid (9:9:1, by vol.); J, ethyl acetate/methanol/acetic acid (15:5:1, by vol.).

Liquid ion-exchangers (Mattox *et al.*, 1972, 1975a,b) were used for chromatography on paper and columns. For paper the solvent systems were: (K) 0.05 M-Aliquat (in chloroform)/0.05 M-KCl (in formamide); (L) 0.1 M-tetraheptylammonium acetate (in chloroform)/0.1 M-potassium acetate (in formamide); (M) 0.1 M-tetraheptylammonium acetate (in chloroform)/0.4 M-potassium acetate (in formamide); (N) 0.05 M-KCl (in water)/0.05 M-Aliquat (in chloroform); (O) 0.1 M-KCl (in water)/0.05 M-Aliquat (in chloroform); (P) 0.2 M-KCl (in water)/0.05 M-Aliquat (in chloroform). Systems K-M are essentially of chloroform/formamide with an exchanger dissolved in the mobile phase and a counterion in the stationary phase. For systems N-P a sheet of paper was drawn slowly through 0.05 M-Aliquat dissolved in chloroform, and suspended from a glass rod until the chloroform had evaporated (approx. 5 min). The conjugates were applied in methanol,

and the paper was placed in a pre-equilibrated jar, which was lined with wet paper; without further equilibration the chromatogram was developed with aq. KCl of the concentration indicated.

Column chromatography with liquid ion-exchangers in the reverse phase was performed by placing 100g of Celite 545 and 2.5g of Silicone Gum Rubber in 500ml of dichloromethane and evaporating the mixture to complete dryness at 40°C in a rotary evaporator at approx. 3330Pa (25mmHg pressure). An appropriate portion of the rubber-coated Celite was mixed thoroughly with 20% of its weight of Aliquat by using a spatula. For system N, the material was then added to about 10 times its weight of 0.05M-KCl and the mixture was placed *in vacuo* in a rotary evaporator until the coated Celite sank and bubbles ceased to escape. The coated Celite was then added in batches to a column that contained 0.05M-KCl (de-gassed in a rotary evaporator) and packed with a Martin packer. Glucosiduronate(s) was added in 0.05M-KCl and the column was run in the conventional manner. Solvent systems O and P were prepared as above except that 0.10M- and 0.20M-KCl respectively were used.

Natural and synthetic steroids were de-salted by using Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.). The void volume (V_0) in ml of these columns approximates the weight of resin in g. Samples were applied in 0.1M-acetic acid, the resin was washed with 2 V_0 of 0.1M-acetic acid, with 4 V_0 of water, and the steroid was eluted with 8 V_0 of ethanol.

Hydrolysis of steroid conjugates by acid (HCl) was carried out at pH1.0 and 22°C for 24h. β -Glucuronidase (EC 3.2.1.31) was standardized against phenolphthalein glucuronide (Levy & Marsh, 1959); hydrolyses of steroidal glucosiduronates were performed in 0.1M-sodium acetate buffer at pH4.8 and 37°C. Incubations (72h) were followed by percolation of the reaction mixtures through a column of Amberlite XAD-2 (2g), washing with water, and elution of the conjugates and free steroids with ethanol. After evaporation of the ethanol, the residue was partitioned between 2ml of dichloromethane and 2ml of water. The steroid from the organic phase was dissolved in ethanol and quantified by radioactivity and/or by measuring absorbance, in association with an appropriate blank, and by using a molar extinction coefficient of 16200 and the appropriate mol.wt.

Reference samples of aldosterone and 17-isoaldosterone [m.p. 200–202°C, light-absorption max. 239nm in ethanol (ϵ 16300), $\nu_{\max}$. (KBr) 1740, 1720, 1660 and 1620cm⁻¹] were prepared by alkaline hydrolysis of aldosterone 21-acetate (I) by using the procedure described by Schmidlin *et al.* (1957) to hydrolyse ($\pm$)-aldosterone 21-acetate. Column chromatography in system C (Grose *et al.*, 1973)

separated aldosterone and 17-isoaldosterone; the compounds were crystallized from acetone/ether and benzene/cyclohexane respectively.

Preparations

Methyl 2-O-(21-acetoxy-11 β ,18-epoxy-3,20-dioxopregn-4-en-18-yl)-1,3,4-tri-O-acetyl- α -D-glucopyranuronate (II). A mixture of aldosterone 21-acetate (2.415g, 6.0mmol) and CdCO₃ (10.34g, 60mmol; Fisher Scientific Co., Fair Lawn, NJ, U.S.A.) was stirred in benzene (170ml) in the dark as the volume was reduced to 150ml by distillation to remove moisture. A solution of methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy- α -D-glucuronate (14.25g, 36mmol) dissolved in benzene (120ml) was added during 1h and distillation was continued for 3h, the volume of the solution being maintained constant by addition of benzene. The solution was filtered, evaporated, and chromatographed on a column (9.5cm $\times$ 43cm) in system A. Material eluted at 4.5 V_0 was rechromatographed on a column (9.5 $\times$ 39cm) in system D. Compound (II) was eluted at 5.0 V_0 and compound (IV) [see preparation of compound (IV) from compound (I)] at 7.7 V_0 . Compound (II) is formulated tentatively as methyl 2-O-(21-acetoxy-11 β ,18-epoxy-3,20-dioxopregn-4-en-18-yl)-1,3,4-tri-O-acetyl- α -D-glucuronate. Compound (II) was crystallized from ethanol/water (yield: 431mg; 10%) and had m.p. 135–139°C [m.p. reported by Underwood & Frye (1972): 125–130°C], $[\alpha]_D^{20} +202 \pm 2^\circ$ (in chloroform), light-absorption max. in ethanol 239nm (ϵ 16300) and $\nu_{\max}$. (KBr) 1735, 1730 (shoulder), 1670, 1616 and 1215cm⁻¹. Table 1 gives values from the n.m.r. spectrum (Found: C, 59.8; H, 6.4; OMe, 4.2. Calc. for C₃₆H₄₆O₁₅: C, 60.0; H, 6.5; OMe, 4.3%). Slightly lower yields of compounds (II) and (IV) were obtained when Ag₂CO₃ was used instead of CdCO₃ in the foregoing reaction.

Attempts to remove the acetate groups from compound (II) by transesterification in methanolic NaOH (0.01M) and obtain a crystalline product were unsuccessful. The solution became bright yellow on addition of alkali, and multiple products were formed as indicated by paper and t.l.c. The most abundant product (approx. 20% yield), eluted from a thin-layer plate (system J), underwent hydrolysis at pH1.0 to yield material with chromatographic properties identical with those of 17-isoaldosterone.

Methyl 2-O-(21-acetoxy-11 β ,18-epoxy-3,20-dioxopregn-4-en-18-yl)-1,3,4-tri-O-acetyl- α -D-glucopyranuronate 3,20-disemicarbazone (III). Treatment of compound (II) with semicarbazide, as described for preparation of compound (IX), subsequent column chromatography in system B and separation from methanol/water gave a disemicarbazone (58%)

that had m.p. 210°C (decomp.), light-absorption max. in methanol 269nm (ϵ 32800) and $\nu_{\max}$. (KBr) 3522, 3412, 2962, 1749, 1687, 1568, 1436, 1272 and 1212cm⁻¹ (Found: C, 54.7; H, 6.3; N, 10.0. Calc. for C₃₈H₃₂N₆O₁₅: C, 54.8; H, 6.3; N, 10.0%).

Methyl (18R)-21-acetoxy-11 β ,18-epoxy-3,20-dioxopregn-4-en-18-yl 2,3,4-tri-O-acetyl- α -D-glucopyranosiduronate (IV). (A) From aldosterone 21-acetate (I). Compound (IV), eluted from the column at 7.7 V₀, as described in the preparation of compound (II) from compound (I), was crystallized from ethanol/water (yield: 1.55g; 36%) and had m.p. 201–202°C, $[\alpha]_D^{20} +230 \pm 2^\circ$ (in chloroform), light-absorption max. in ethanol 239nm (ϵ 16500) and $\nu_{\max}$. (KBr) 1753, 1726, 1664, 1617 and 1205cm⁻¹. Data for n.m.r. are given in Table 1. (Found: C, 60.0; H, 6.5; OMe, 4.4. Calc. for C₃₆H₄₆O₁₅: C, 60.0; H, 6.5; OMe, 4.3%).

(B) From β -D-glucosiduronate (VIII). A portion (2ml; approx. 0.025mmol steroid) of the suspension of reactants described for the synthesis of compound (VIII) from compound (I) was removed immediately after the acetobromoglucuronate had been added; this 2.00ml portion was added to 345 μ g of [³H] β -D-glucosiduronate (VIII), which was derived from the ³H-labelled urinary glucosiduronic acid (XII). The solution was stirred for 20h in the dark, filtered, evaporated and the product was chromatographed on a column by using system A. The band eluted at 7.1 V₀ contained 77% of the tracer added and that eluted at 20.0 V₀ [β -glucosiduronate; (VIII)] contained 22% (a recovery of 99% of the 50000d.p.m. added to the mixture at the initiation of the Koenigs-Knorr reaction). The product corresponding to 7.1 V₀ was rechromatographed (system D) and a crystalline substance that contained ³H and was identical with compound (IV) (m.p., mixed m.p., i.r., and chromatography in systems A, D and G) was obtained. Thus under these experimental conditions β -D-glucosiduronate (VIII) is converted into α -D-glucosiduronate (IV) to the extent of approx. 77%.

(C) From aldosterone α -D-glucopyranosiduronic acid (VI). Compound (VI) (32mg; 0.060mmol) dissolved in methanol was esterified with diazomethane, the product was acetylated in 0.5ml of pyridine and 0.5ml of acetic anhydride for 24h, and crystals (42mg) were obtained from ethanol/water and identified (m.p., i.r. and chromatography) as compound (IV).

Methyl (18R)-11 β ,18-epoxy-21-hydroxy-3,20-dioxopregn-4-en-18-yl α -D-glucopyranosiduronate (V). Esterification of compound (VI) (100mg, 0.18mmol) with diazomethane followed by chromatography of the product on a column (2.5cm $\times$ 33cm) of silicic acid in chloroform/ethanol (2:1, v/v) yielded a peak at 3.9 V₀ that gave 86mg (yield: 66%) of an amorphous solid (V) from acetone/benzene with

m.p. 156–159°C, light-absorption max. in ethanol at 239nm (ϵ 16300), and $\nu_{\max}$. (KBr) 3405, 1751, 1712, 1666 and 1615cm⁻¹ (Found: C, 61.2; H, 7.1; OMe, 5.7. Calc. for C₂₈H₃₈O₁₁: C, 61.1; H, 7.0; OMe, 5.6%).

(18R)-11 β ,18-Epoxy-21-hydroxy-3,20-dioxopregn-4-en-18-yl α -D-glucopyranosiduronic acid (VI). Compound (IV) (300mg, 0.42mmol) was allowed to stand at room temperature (23°C) in 8ml of 0.033M-NaOH in chloroform/methanol (1:4, v/v) for 5h. The pH was adjusted to 4.0 with dil. H₂SO₄, and the solution was filtered and evaporated to remove solvents. The volume was adjusted to 40ml with water and the solution was passed through 40g of Amberlite XAD-2 to remove inorganic material. The product was chromatographed on a column (2.5cm $\times$ 37cm) by using system E. The major peak (4.8 V₀) yielded crystalline compound (VI) (yield: 190mg; 84%) from methanol/water with m.p. 234–237°C, $[\alpha]_D^{20} +272 \pm 2^\circ$ (in methanol), light absorption max. in methanol 240nm (ϵ 16300), and $\nu_{\max}$. (KBr) 3400, 1748, 1710, 1665 (shoulder), 1621 and 1610 (shoulder) cm⁻¹ (Found: C, 60.3; H, 6.9. Calc. for C₂₇H₃₆O₁₁: C, 60.4; H, 6.7%). At pH1.0, this product was hydrolysed to yield aldosterone (Table 4).

(18R)-11 β ,18-Epoxy-21-hydroxy-3,20-dioxo-17 α -pregn-4-en-18-yl α -D-glucopyranosiduronic acid (VII). A minor peak, eluted at 7.5 V₀ from the chromatogram described in the previous paragraph, gave an amorphous solid (yield: 32mg; 12%) from methanol/ethyl acetate with m.p. 200°C (decomp.), $[\alpha]_D^{20} +124 \pm 2^\circ$ (in methanol), light-absorption max. 240nm (ϵ 16300) and $\nu_{\max}$. (KBr) 3364, 1721 (broad), 1660 and 1620 (shoulder) cm⁻¹ (Found: C, 60.1; H, 7.0. Calc. for C₂₇H₃₆O₁₁: C, 60.4; H, 6.8%). On hydrolysis at pH1.0 this material gave 17-isoaldosterone, i.e. the 18,11-hemiacetal of 11 β ,21-dihydroxy-3,20-dioxo-17 α -pregn-4-en-18-al.

When 0.01M-NaOH in methanol was used for transesterification of compound (IV) and this treatment was followed by hydrolysis of the methyl ester in aq. 0.10M-NaOH, the yield of the aldosterone α -D-glucosiduronic acid (VI) was 50% and that of 17-isoaldosterone α -D-glucosiduronic acid (VII) was 46%.

11 β -Hydroxy-3-oxo-18-norandrost-4-en-13,17 α -dicarboxylic acid γ -lactone methyl ester. Treatment of 17-isoaldosterone (75mg) with 0.10M-NaIO₄ solution, esterification of the product with diazomethane and oxidation of the C-18 hydroxyl group with CrO₃ by the general procedures of Simpson *et al.* (1954) gave 53mg (yield: 76%) of crystalline product from ether with m.p. 219–222°C, light-absorption max. in methanol 239nm (ϵ 16300), and $\nu_{\max}$. (chloroform) 1771, 1730, 1665 and 1617cm⁻¹ (Found: C, 70.4; H, 7.6. Calc. for C₂₁H₂₇O₅: C, 70.2; H, 7.6%). The racemic 11 β -hydroxy-3-oxo-18-norandrost-4-ene-13,17 α -dicarboxylic acid γ -lactone methyl ester,

Table 4. *Acid- and enzyme-catalysed hydrolysis of steroidal glucosiduronic acids*

Hydrolyses were performed as described in the text. For each reaction approx. 0.3 μ mol of conjugate was used in a total vol. of 3.04 ml. Values in the table give the mean percentage yield of free steroid from triplicate determinations. Aldo β -GA, aldosterone 18- β -D-glucopyranosiduronic acid; Aldo α -GA, aldosterone 18- α -D-glucopyranosiduronic acid; E β -GA, cortisone 21- β -D-glucopyranosiduronic acid; A β -GA, 11-dehydrocorticosterone 21- β -D-glucopyranosiduronic acid.

Treatment	Compound	Yield (%)				
		Aldo β -GA (synthetic) (XII)†	Aldo β -GA (natural) (XII)†	17-Iso- Aldo β -GA* (XIII)†	E β -GA	Aldo α -GA (synthetic) (VI)‡
pH 1.0		92	92	71	6	81
Acetate buffer		4	3	4	3	4
Ketodase						
1000 U§		4	5			
3500 U		25	24	25	77	9
10000 U		32	36			
Sigma B-1						
1000 U		52	46			
3500 U		80	84		72	21
<i>H. pomatia</i>						
1000 U		29	38			
3500 U		74	76	71	76	74

* Derived from 3 H-labelled urinary Aldo β -GA (XII).

† See Scheme 3.

‡ See Scheme 1.

§ U = Fishman units of β -glucuronidase.

which was prepared by Wieland *et al.* (1958), has spectral properties comparable to those of the foregoing substance, but has a m.p. of 199–201.5°C.

Methyl 21-acetoxy-11 β ,18-epoxy-3,20-dioxopregn-4-en-18-yl 2,3,4-tri-O-acetyl- β -D-glucopyranosiduronate (VIII). (A) From aldosterone 21-acetate (I). A mixture of aldosterone 21-acetate (1.206 g, 3.0 mmol), Ag₂CO₃ (16.55 g, 60 mmol; Wolfrom *et al.*, 1961) and anhydrous CaSO₄ (10 g) was stirred in dry benzene (150 ml) for 20 min and methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy- α -D-glucuronate (22.88 g, 60 mmol; Bollenback *et al.*, 1955) dissolved in benzene (75 ml) was added during 20 min [a portion (2 ml) was removed to prepare compound (IV) from compound (VIII)]; stirring was continued for 2 h in the dark. The solution was filtered and evaporated. Essentially no unchanged starting material (I) remained. Chromatography of the residue on a column (5 cm $\times$ 30 cm) in system A gave a band at approx. 4.5 V_0 that contained a mixture of compounds (II) and (IV) (combined yield approx. 60%) that was not separated; an additional band at 12.5 V_0 contained compound (VIII), which was rechromatographed on a column (2.5 cm $\times$ 26 cm) by using system D. A band eluted at 12.5 V_0 yielded crystals (yield: 202 mg; 9.5%) of compound (VIII) from acetone/water, with m.p. 185–187°C with spontaneous recrystallization and remelting at 224–225°C, $[\alpha]_D +115 \pm 2^\circ$ (in chloroform), light-absorption max. in ethanol 239 nm (ϵ 16200), and $\nu_{\max}$ (KBr) 1766, 1736, 1666, 1616 and 1230 cm⁻¹. For n.m.r. data see Table 1. (Found:

C, 59.9; H, 6.4; OMe, 4.5. Calc. for C₃₆H₄₆O₁₅: C, 60.0; H, 6.5; OMe, 4.3 %.) This product is identical (mixed m.p., i.r. spectrum and n.m.r. spectrum) with the product of m.p. 184–187°C and 223–225°C that was prepared from urinary glucosiduronic acid (XII).

The detection of compound (VIII) in the product from the Koenigs–Knorr reaction was first achieved when the reaction was run on a 1.0 mmol scale under conditions essentially the same as above, but for a longer time. After the silver salts had been removed and the solution had been evaporated, 100000 d.p.m. (approx. 675 μ g) of compound (VIII) [derived from 3 H-labelled compound (XII)] was added to the residue and the product was chromatographed as above. A band at 4.5 V_0 contained 96% of the added radioactivity. Chromatography of this product in system D gave a band at 12.5 V_0 that contained 93% of the initial radioactivity and yielded 42 mg of compound (VIII), with m.p. 187–188°C and 224–225°C.

(B) From urinary glucosiduronic acid (XII). To a methanolic solution of 3 H-labelled acid-labile conjugate (XII) (11.1 mg, 20.8 μ mol, specific radioactivity 1.06×10^{11} d.p.m./mol) in the carboxylic acid form was added a sixfold excess of diazomethane in ether. After 20 min, an excess of dilute acetic acid was added, the solvent was evaporated, and the residue was dissolved in 0.4 ml of pyridine and 0.4 ml of acetic anhydride. After 16 h, water was added, the product was recovered and chromatographed on a column (0.9 cm $\times$ 44 cm) in system D. Fractions of

eluate were monitored for u.v. absorbance, Blue Tetrazolium reduction and radioactivity. Material eluted at 12.8 V_0 was pooled and crystals (yield: 10.2mg; 65%) were obtained from acetone/water; the product (VIII) melts at 184–187°C, reorganizes spontaneously to larger crystals, and remelts at 223–225°C and has light-absorption max. in ethanol 239nm (ϵ 16300), specific radioactivity 1.06×10^{11} d.p.m./mol, and $\nu_{\max}$. (KBr) 1768, 1738, 1715 (shoulder), 1666, 1616 and 1230 cm^{-1} . N.m.r. data are included in Table 1. (Found: C, 60.1; H, 6.5. Calc. for $\text{C}_{36}\text{H}_{46}\text{O}_{15}$: C, 60.0; H, 6.5%.)

Methyl 21-acetoxy-11 β ,18-epoxy-3,20-dioxopregn-4-en-18-yl 2,3,4-tri-O-acetyl- β -D-glucopyranosiduronate 3,20-disemicarbazone (IX). To a solution of tetra-acetyl ester (VIII) (290mg, 0.40mmol) in chloroform/methanol (20ml, 1:3, v/v) was added NaHCO_3 (407mg, 4.84mmol) and semicarbazide hydrogen chloride (900mg, 8.06mmol) in 3ml of water. The solution was maintained at 50°C for 28h, the solvent was evaporated and the residue was partitioned between chloroform and water. The organic extract was chromatographed on a column (3.6cm $\times$ 22.5cm) in system B. A peak eluted at 1.6 V_0 contained a product that, based on its chromatographic mobility and its light-absorption maximum of 269nm, is thought to be the C-3 monosemicarbazone of compound (II); a peak at 7.7 V_0 gave 211 mg (yield: 63%) of partially crystalline disemicarbazone (IX) from chloroform/ethanol, with m.p. 195°C (decomp.), light-absorption max. in ethanol 269nm (ϵ 32300), and $\nu_{\max}$. (KBr) 3482, 1761, 1686, 1537 and 1228 cm^{-1} (Found: C, 54.6; H, 6.3. Calc. for $\text{C}_{38}\text{H}_{32}\text{N}_6\text{O}_{15}$: C, 54.8; H, 6.3%).

Retreatment of the C-3 monosemicarbazone with semicarbazide yielded an additional 27mg of compound (IX) (yield: 8%) for a total yield of 71%.

Methyl 11 β ,18-epoxy-21-hydroxy-3,20-dioxopregn-4-en-18-yl β -D-glucopyranosiduronate (X). Synthetic acid (XII) (48mg; 0.09mmol) dissolved in methanol was treated with an excess of diazomethane in diethyl ether. After 20min an excess of acetic acid was added, the solvent was removed and the residue, which had approx. 10% impurity based on t.l.c. in system J, was chromatographed on a column (1.5cm $\times$ 26cm) by using system F. The band eluted at 9 V_0 gave 42mg (yield: 87%) of amorphous solid (X) from chloroform/cyclohexane, with m.p. 113–116°C, light-absorption max. in ethanol 239nm (ϵ 16400), and $\nu_{\max}$. (KBr) 3440, 1750, 1705, 1667 and 1615 cm^{-1} (Found: C, 61.0; H, 7.2. Calc. for $\text{C}_{28}\text{H}_{38}\text{O}_{11}$: C, 61.0; H, 7.0%).

Isolation of the acid-labile conjugate of aldosterone from urine (XII). After obtaining approval from the Human Studies Committee, Mayo Clinic, and after obtaining informed consent from paid healthy human volunteers, each individual was given 150mg of aldosterone by mouth; one individual received, in addition,

65 μCi of [1,2,6,7- ^3H]aldosterone, which had been deposited on to sugar cubes by evaporation from a stock solution in benzene/ethanol. Each subject collected urine for 12h in a bottle that contained 10mg of disodium EDTA. The urine from the four volunteers was combined, the volume was measured and portions were removed for the determination of radioactivity.

The combined urine was made 0.10M with acetic acid and percolated through 600g of activated (Bradlow, 1968) Amberlite XAD-2 in a column (5cm $\times$ 45cm) (Scheme 2). The column was washed successively with 1 litre of 0.1M-acetic acid, 4 litres of water, and 4 litres of ethanol. The acidic and aqueous effluents contained less than 1% of the total radioactivity. The residue from the ethanolic eluate was dissolved in 200ml of water and the solution was extracted twice with 50ml of chloroform. The organic extract contained approx. 1% of the total ^3H . After the volume of the aqueous phase had been reduced to 150ml, the product was chromatographed on a column (3.6cm $\times$ 42cm) of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) essentially as described by Grose *et al.* (1973). Fractions (50ml) were collected during elution with 1000ml of water, with 1250ml of 0.12M-NaCl, and with 1250ml of 0.25M-NaCl (Scheme 2). The material in fractions 23–45 contained steroidal glucosiduronates and represented 85% of the total radioactivity from the urine. These fractions were combined, the solution acidified with acetic acid and passed through a column of Amberlite XAD-2 (350g) to remove the NaCl. The ethanolic eluate was evaporated and the residue was chromatographed on a column (5cm $\times$ 35cm) by using system E. Material eluted at 4.4 V_0 contained the acid-labile conjugate and was combined and evaporated after addition of 10 μg disodium EDTA. [The material eluted at 8.8 V_0 contained the glucosiduronate of tetrahydroaldosterone (Alvarez *et al.*, 1976)]. The acid-labile conjugate was then chromatographed by reverse-phase technique (system P) on a column (2.5cm $\times$ 42cm) with Aliquat as ion exchanger and aq. 0.2M-KCl as mobile phase. The material that emerged at 6.2 V_0 was extracted with dichloromethane (to remove a trace of ion exchanger from the mobile phase) and the aq. solution was desalted on 40g of Amberlite XAD-2. The combined ethanolic eluate was evaporated after addition of 1 μg of disodium EDTA.

The absorption of the ethanolic eluate at 239nm (assuming ϵ = 16200) corresponded to 57mg of acid-labile conjugate (XII) and represented a yield of approx. 7% from the orally ingested aldosterone. The specific radioactivity was 9.87×10^{10} d.p.m./mol; the calculated specific radioactivity of the combined oral doses was 8.67×10^{10} d.p.m./mol. The 57mg of material was homogeneous in multiple chromatographic systems (E, I, K and O), and had light-

absorption max. in methanol 240nm and $[\alpha]_D^{25} +154 \pm 2^\circ$ (in methanol). This product released aldosterone when incubated at pH 1.0 and was resistant to β -glucuronidase (Ketodase) hydrolysis as shown in Table 4. This compound (XII) is the acid-labile conjugate of aldosterone. In an additional experiment in which slightly less unlabelled aldosterone was ingested, the specific radioactivity of the conjugate that was isolated was 1.06×10^{11} d.p.m./mol.

In a previous similar experiment homogeneous acid-labile conjugate (XII) was isolated without EDTA; however, after standing in methanol for 24h about half of the conjugate had been converted into a product that gave a positive reaction with the Porter-Silber reagent. The impurity and compound (XII) could be separated by paper chromatography in system E. Probably the Porter-Silber positive product is the 21-aldehyde of compound (XII), and is formed by oxidation of compound (XII) by Cu^{2+} , which can be leached from the Celite during chromatography (Mattox & Vrieze, 1972).

Synthetic 11 β ,18-epoxy-21-hydroxy-3,20-dioxo-pregn-4-en-18-yl β -D-glucopyranosiduronic acid (XII). The disemicarbazone (IX) (211 mg, 0.254 mmol) was transesterified with 0.10M-NaOH dissolved in dry chloroform/methanol (8ml; 1:1, v/v) during 30 min to give an intermediate (XI). Methanol (8ml) and aq. 1.0M-NaOH (4ml) were added; hydrolysis of the methyl ester was complete within 30 min. The pH was adjusted to 3.4 with dil. H_2SO_4 to give the intermediate (XIV), water was added, and the organic solvents were removed. The aq. solution was percolated through 40g of Amberlite XAD-2 to remove inorganic material. The intermediate (XIV) was eluted with ethanol, the solution was evaporated and the residue was dissolved in 60ml of methanol/water (80:20, v/v). After adjustment to pH 2.0 with dil. H_2SO_4 , the solution was left for 90 min during which time the semicarbazone groups were removed. The volume was reduced to 50ml, the conjugate was absorbed on Amberlite XAD-2 (40g) and the column was washed with 5mM- H_2SO_4 (80ml), water (three 80ml portions) and ethanol (four 80ml portions). The residue from the alcoholic eluate was chromatographed on a column (2.5cm $\times$ 42cm) using reverse-phase technique with 20% (w/w) Aliquat stationary phase and 0.20M-KCl as mobile phase. The eluate which appeared at 5 V_0 was desalted on Amberlite XAD-2. Based on a formula mol.wt. of 536.6 and ϵ of 16200, the absorbance of the residue in ethanol at 239nm corresponded to 92mg of compound (XII) [66% yield from compound (IX)]. This product was divided into two portions. Efforts to crystallize one-half the residue as the acid, the ammonium salt, the potassium salt and the barium salt were unsuccessful. The other portion yielded 46mg of tractable amorphous solid on slow evaporation of a solution in acetone/ethyl acetate,

with m.p. 101–103°C, bubbles at 94°C, $[\alpha]_D^{25} +152 \pm 2^\circ$ (in methanol), light-absorption max. in methanol 240nm, and ν_{max} (KBr) 3450–3350, 1704, 1666 and 1614 cm^{-1} . In chromatography systems E, I, K, L and N this product migrated at the same rate as glucosiduronic acid (XII), which was isolated from urine. The presence of a carboxyl function in compound (XII) was demonstrated by dissolving 1.0mg of the compound in methanolic 3.0mM- NH_3 , removing the solvent and taking the i.r. spectrum in KBr; the product had a strong band at 1610 cm^{-1} , a frequency at which compound (XII) had only the weak band of C=C.

11 β ,18-Epoxy-21-hydroxy-3,20-dioxo-17 α -pregn-4-en-18-yl β -D-glucopyranosiduronic acid (XIII). A methanolic solution of synthetic acid-labile conjugate (XII) (2mg) was adjusted to pH 10.0 with aq. 1M-NaOH. The solution was left at 22°C for 30 min, the pH was adjusted to 3.0 with acetic acid and the mixture was desalted on Amberlite XAD-2. In chromatography systems E, K and O the product migrated at a rate different from that of compound (XII) and produced a single u.v.-absorbing spot, which gave a positive test with alkaline Tetrazolium Blue. Hydrolysis of the product at pH 1.0 with aq. HCl yielded 17-isoaldosterone, which was identified by its chromatographic properties and i.r. spectrum. The natural acid-labile conjugate (XII), labelled with ^3H , was converted into the 17-iso conjugate (XIII) by the same procedure and the product was identified similarly.

Timed treatment of compound (VIII) with methanolic NaOH (0.01M and more dilute solutions) followed by (a) chromatographic comparison of the product with compound (X) and (b) hydrolysis of the product at pH 1.0 and chromatographic comparison of the free steroid(s) with aldosterone and 17-isoaldosterone showed that extensive inversion at C-17 had occurred by the time the acetyl groups had been removed from compound (VIII). Treatment of compound (VIII) under conditions described for preparation of compound (VI) from compound (IV) gave almost exclusively the 17-iso derivative (XIII).

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that the analysis shows the asymmetric centre at C-18 to have the *R* configuration. This work was supported by grant AM-05452 from the NIH, USPHS, and by a grant from the Northwest Area Foundation, St. Paul, MN, U.S.A. P. C. C. was supported in part by a grant AM-5628 from NIH, USPHS.

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