Synthesis of C-21 Glucosiduronates of Cortisone and Related Corticosteroids*

Vernon R. Mattox, June E. Goodrich, and Wiley D. Vrieze

ABSTRACT: On treatment with methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate, cortisone yielded the corresponding 21-glucosiduronate triacetate methyl ester. Removal of the acetyl groups from the conjugate by transesterification with sodium hydroxide in methanol produced the corresponding methyl glucosiduronate. Hydrolysis of the methyl ester linkage with sodium hydroxide followed by acidification produced cortisone-21-glucosiduronic acid. The methyl triacetyl ester of cortisone-21-glucosiduronic acid formed a 3,20-bissemicarbazone which was reconverted into the 3,20-dioxo derivative by treatment with pyruvic acid. Analogous glucosiduronate derivatives have been synthesized from cortisol, 11-deoxycortisol, corticosterone, 11-dehydrocorticosterone, and 11-deoxycorticosterone. Steroidal glucosiduronic acids can be separated from inorganic salts and acids by adsorption on a polystyrene resin (Amberlite XAD-2) which does not adsorb the inorganic components; the glucosiduronic acids can be eluted from the resin by use of ethanol.

Many steroids are excreted in urine as conjugates[1] with glucuronic acid. In most investigations concerning these conjugates, purified extracts have been treated with β-glucuronidase to hydrolyze the glycosidic bonds, and the structures of the conjugates have been deduced from study of the structures of the products (Pasqualini, 1963; Schneider and Lewbart, 1959). In a few instances a conjugate has been isolated in crystalline form and identified by comparison with a synthetic steroidal glucosiduronate of known structure (for example, Foggitt and Kellie, 1964; Hashimoto and Neeman, 1963; Heard *et al.*, 1944; Huebner *et al.*, 1944; Schneider *et al.*, 1955).

Conjugates of cortisone (Nitta *et al.*, 1964; Wotiz *et al.*, 1959; Zorbach and Valiaveedan, 1964), of cortisol and 11-deoxycortisol (Nitta *et al.*, 1964), and of 11-deoxycorticosterone (Pelzer, 1959; Zorbach, 1958) with glucuronic acid have been prepared as the methyl tri-*O*-acetyl esters. After hydrolysis, however, it was not possible to obtain the corresponding glucosiduronic acids in crystalline form.

This paper describes preparation of crystalline C-21 glucosiduronic acid derivatives of cortisone, cortisol, 11-deoxycortisol, corticosterone, 11-dehydrocorticosterone, and 11-deoxycorticosterone *via* the intermediates shown in Figure 1. In general, experimental conditions were sought which would give a satisfactory yield of the various derivatives of cortisone, and these conditions were then applied to the preparation of the analogous derivatives of the other steroids.

To synthesize substances IIa–f, we used the Meystre–Miescher (1944) modification of the Koenigs–Knorr reaction. Methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate was coupled with the appropriate steroid in benzene in the presence of freshly prepared dry silver carbonate (McCloskey and Coleman, 1955).

Because it was not possible to crystallize the conjugates (IIa–f) in reasonable purity and yield from the reaction mixtures, the solutions were subjected to column chromatography. The eluates were monitored by treating aliquots of the effluent (on strips of paper) with alkaline tetrazolium blue and by testing for sodium hydroxide induced fluorescence. These test procedures indicated that the principal product from each starting material was the conjugate with a glucosiduronate group at C-21 of the steroid.

To obtain the maximal yield of conjugate IIa from cortisone (Ia), it is necessary to use an excess of the bromo compound (Bollenback *et al.*, 1955) because this substance, as previously found for 2,3,4,6-tetra-*O*-acetyl-1-bromo-1-deoxy-α-D-glucose (Goldschmid and Perlin, 1961), is unstable in hot benzene in the presence of silver carbonate. When 1 mmole of cortisone was treated with 2 equiv of bromo compound, a yield of 44% of conjugate IIa was obtained along with about 10% of unchanged cortisone. With 3 equiv of bromo intermediate the yield of IIa was 53% and about 1.5% of unchanged cortisone remained; several minor by-products, which were not isolated in crystalline form, were present.

Compound IIe, the methyl triacetyl ester of 11-dehydrocorticosterone-21-glucosiduronic acid, was prepared by oxidation of the corresponding 11β-hydroxy compound (IId) with chromic oxide in pyridine rather than by coupling the free steroid itself and the bromo glucuronic ester. The oxidized product (IIe) was ob-

* From the Mayo Clinic and Mayo Foundation, Section of Biochemistry, Rochester, Minnesota. *Received April 17, 1968.* This investigation was supported in part by Research Grant AM-5452 from the National Institutes of Health, Public Health Service.

[1] Simplified names are used for the conjugates in the narrative section of the paper; systematic names are used in the Experimental Section. For nomenclature of glucosiduronates see *J. Org. Chem. 28*, 281 (1963).

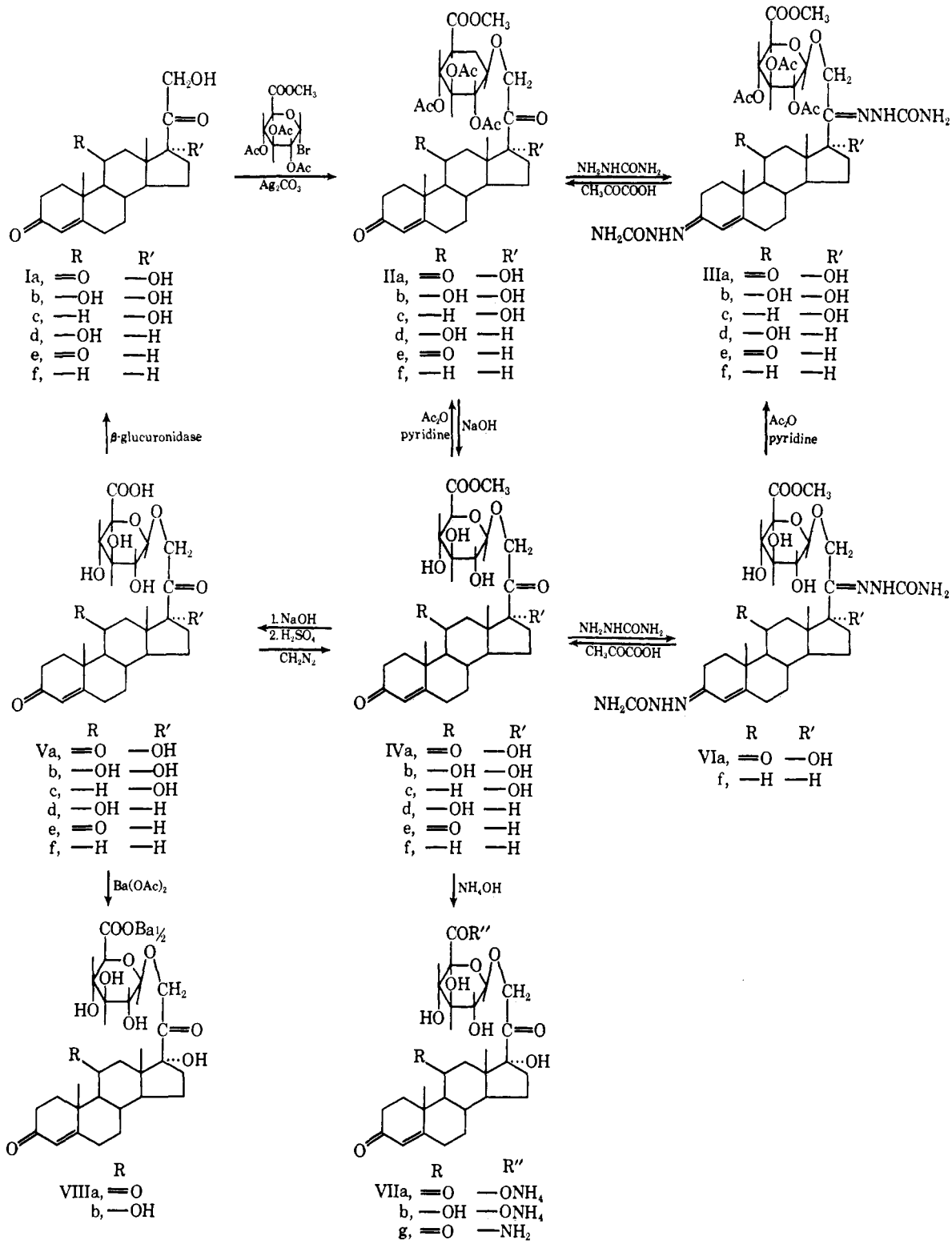

FIGURE 1: Synthesis of C-21 glucosiduronates of cortisone and related corticosteriods.

tained from IId in 87% yield. Compound IIa could be prepared from IIb by a similar procedure.

That products IIa–f are C-21 glucosiduronates is indicated by the finding that they do not reduce alkaline tetrazolium blue and are not altered by mild treatment with acetic anhydride–pyridine. In addition, compounds IIe and IIf have no hydroxyl band in the infrared spectrum. The possibility that the substances are C-3 or C-20 enol glucosiduronates is ruled out by formation of the bissemicarbazones (IIIa–f). That the C-11

oxygen function is not involved in the glycosidic linkage is indicated by preparation of IIa from IIb and of IIe from IId by oxidation with chromic oxide.

The evidence in the following paragraphs indicates that the glycosidic linkage at C-21 has the β configuration. Experience with the synthesis of a large number of glycosides by use of the Koenigs–Knorr reaction (Conchie *et al.*, 1957) has shown that, with silver carbonate as the coupling agent, formation of the β-glycoside is almost the invariable rule.

The nuclear magnetic resonance spectrum of cortisone-21-glucosiduronic acid, as the triacetate ester (IIa), is compatible with the β configuration for the glycosidic linkage and with the structural formula of IIa. These spectral findings are in general accord with those of Neeman and Hashimoto (1962) for methyl (3-methoxy-17β-acetoxyestra-1,3,5(10)-trien-16α-yl 2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate, a β-glycosidic compound which was derived from urinary estriol-16-glucosiduronic acid and was prepared synthetically (Carpenter and Kellie, 1962) by a procedure using the Koenigs–Knorr reaction.

Neeman and Hashimoto (1962) observed that all three acetoxyl groups of the glucuronyl moiety of their estriol conjugate gave a single band. However, in the spectrogram of our compound IIa, one acetoxyl group absorbs at a lower field than the others. Selective deshielding of one acetoxyl group has been observed in similar situations (Lemieux *et al.*, 1958) and thus the deshielding position of one of the three groups does not necessarily preclude the all-equatorial conformation expected of the β-D-glucuronic acid derivative.

Four lines on the spectrogram of IIa, at δ 4.15, 4.46, 4.69, and 4.99 ppm with a coupling constant of 17 cps may be attributed to the C-21 methylene group of the glycosidic linkage. This four-line AB pattern is present in the spectrogram of 21-acetoxy-20-oxo steroids (Shoolery and Rogers, 1958) and has a coupling constant of about 16 cps. These lines do not appear to be present in the spectrograms determined by Neeman and Hashimoto (1962) on compounds which lack the side chain of a corticosteroid.

Overlapping with the C-21 methylene group are bands at 4.60 and 4.72 ppm with a peak separation of about 7 cps. These bands are ascribable to the 1 proton of the glucuronyl moiety in a *trans*-diaxial relationship. The chemical shift of this doublet is 4.66 ppm. For the corresponding shift and coupling constant of the 1′ proton of methyl (3-methoxy-17β-acetoxyestra-1,3,5-(10)-trien-16α-yl 2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate, Neeman and Hashimoto (1962) reported 4.50 ppm and 7 cps. Thus the nuclear magnetic resonance spectrogram of IIa indicates that the glycosidic linkage of this substance is β oriented.

The nuclear magnetic resonance spectrogram of cortisone-21-glucosiduronic acid (Va) in deuterated dimethyl sulfoxide is very complicated and displays no clear evidence to confirm or deny the existence of a β-glycosidic linkage.

In a study of the infrared spectra of anomeric glycosides, Barker *et al.* (1954) found an absorption peak of moderate to strong intensity at 844 ± 8 cm^{-1} for all α anomers examined; the β anomers lacked this peak but had a band at 891 ± 7 cm^{-1}. In a later paper (Stacey *et al.*, 1958) it was concluded that the bands at 844 and 891 cm^{-1} do not represent C–H deformation vibrations at C-1; nevertheless, these bands are considered to represent vibration of the whole molecule at C-1 and to be dependent on whether the configuration at C-1 is α or β. None of our compounds (IIa–f and Va–f) contained a band in the 844 ± 8 cm^{-1} region;[2] all of the esters (IIa–f) had bands in the range of 891 ± 7 cm^{-1} and all of the acids had bands in the range 869–884 cm^{-1}.

Nitta *et al.* (1961) reported that α anomers of fully acetylated glucopyranuronic acid derivatives have characteristic absorption bands at 1150 and 940 cm^{-1} and that β anomers lack these bands. A more recent paper by Nitta and Ide (1965) confirmed these findings. When Nitta *et al.* (1964) prepared IIa–c, they interpreted the infrared spectra of these conjugates in terms of the foregoing findings and assigned the β configuration to the glycosidic linkage of these compounds. Furthermore, the infrared spectra which we determined on glucosiduronates IIa–f do not have absorption bands at 1150 or 940 cm^{-1}.

The molecular rotations of the α and β anomers of each of the glucosiduronates (IIa–f) may be calculated by adding the molecular rotations of their component parts in the manner described by Klyne (1950) and utilized subsequently by Zorbach (1958) and by Nitta *et al.* (1964) for steroidal glucosiduronates. Comparison of the molecular rotation found for each of the synthetic glucosiduronates (IIa–f) with that calculated for its α and β anomers provides a good basis for assigning configuration to the anomeric center of each synthetic conjugate. As shown in Table I, the molecular rotation found for each synthetic compound is in good agreement with that calculated for the β anomer but in poor agreement with that calculated for the α anomer. These data indicate that the glycosidic linkages in IIa–f are β oriented. This assignment of configuration is confirmed by the finding (V. R. Mattox, J. E. Goodrich, and W. Vrieze, 1968, unpublished data) that β-glucuronidase (Ketodase) will hydrolyze the glycosidic linkage of Va–f.

Treatment of the triacetyl ester glucosiduronates (IIa–f) overnight with semicarbazide in aqueous methanol containing sodium bicarbonate gave the corresponding 3,20-bissemicarbazones (IIIa–f) in good yields. These derivatives, on reaction with pyruvic acid, were reconverted into the corresponding 3,20-dioxo compounds (IIa–f).

Initially, the acetyl groups were removed from conjugates IIa–f by brief treatment with a catalytic amount of sodium methylate in methanol, and the corresponding methyl esters were obtained. Subsequently, equally

[2] The infrared spectra were determined in KBr on a Beckman IR-5A spectrophotometer by Mr. H. Garrett Rosenthal in the laboratory of Dr. Frank Ungar, Department of Biochemistry, University of Minnesota.

TABLE I: Molecular Rotation of Conjugates.

Compd	Calculated (deg)[a]		Found (deg)
	α-Glycoside	β-Glycoside	
IIa	+1359	+653	+590
IIb	+1210	+504	+489
IIc	+1045	+339	+258
IId	+1376	+670	+684
IIe	+1492	+786	+741
IIf	+1192	+486	+439[b]

[a] For Ic, the molecular rotation, M_D, in ethanol is +440° (this paper). M_D values for the other free steroids are calculated from values for specific rotation in ethanol; for Ia (Wintersteiner and Pfiffner, 1936), for Ib and Id (Reichstein, 1937), for Ie (Kuizenga and Cartland, 1939), for If (Steiger and Reichstein, 1937). M_D for methyl (1-methyl 2,3,4-tri-*O*-acetyl-α-D-glucosid)uronate is +605° (Hardegger and Spitz, 1949). M_D for methyl (1-methyl 2,3,4-tri-*O*-acetyl-β-D-glucosid)uronate is −101° (Hardegger and Spitz, 1950). [b] Zorbach, 1958.

good yields were obtained with sodium hydroxide[3] as the catalyst. Esters IVa–e were obtained as well-formed crystals. Ester IVf was prepared in chromatographically pure form but could not be crystallized. A sample of ester IVd, which had been prepared from Vd by treatment with diazomethane, gave crystals from ethyl acetate; however, subsequent attempts to obtain this substance in crystalline form were unsuccessful.

Treatment of the trihydroxy esters (IVa–f) with acetic anhydride in pyridine produced the corresponding triacetyl derivatives (IIa–f); acetylation was almost complete in 30 min at room temperature.

The 3,20-bissemicarbazones of methyl esters IVa and f were prepared by the same procedure used for making semicarbazones IIIa–f from the acetylated conjugates. Acetylation of the hydroxy semicarbazones (VIa and f) gave the respective acetylated semicarbazones (IIIa and f).

In initial experiments, the ester linkage of IVa–f was hydrolyzed by treatment with 1.0 N ammonium hydroxide. This procedure caused formation of a small amount of by-product, tentatively designated as glucosiduronic acid amide,[4] from each of the esters. The amides were discovered when samples of the acids in methanol were converted into the methyl esters by treatment with diazomethane and the products were chromatographed in system S5. Each methyl ester was accompanied by a product which had essentially the same mobility as the corresponding acid but which could be distinguished from the acid by chromatography in system S7 on paper impregnated with 0.1 M barium acetate or lead acetate. The presence of either of these salts greatly retards the movement of the glucosiduronic acid without retarding the movement of the corresponding amide significantly.

Subsequently, the esters were hydrolyzed with dilute sodium hydroxide and the glucosiduronic acids were recovered by acidifying the solution, adding ammonium sulfate, and extracting with butyl alcohol.

Good recovery of Va from its sodium salt, after alkaline hydrolysis of IVa, also was obtained when the solution was acidified with acetic acid, diluted with an equal volume of methanol, and passed through a cation-exchange column in the acid cycle. The presence of methanol in the solution speeds up elution of the conjugate from the resin.

Amberlite XAD-2, a synthetic polystyrene polymer, also has been used in the recovery of glucosiduronic acids Va–f from aqueous salt solutions. This material is commercially available as white 20–50 mesh beads.[5] The resin selectively adsorbs various water-soluble organic compounds from aqueous solution during passage of a solution through the column and allows inorganic substances to emerge in the effluent. The adsorbed organic substances, held to the resin by attraction of the hydrophobic portion of the molecule, are eluted with a mixture of alcohol and water or with alcohol. Use of Amberlite XAD-2 for recovering synthetic steroidal glucosiduronic acids from aqueous media was described in a preliminary report by Mattox and Vrieze (1967); Bradlow (1968) has used the resin to recover conjugates of steroids from urine.

Before use, Amberlite XAD-2 is placed in a chromatography column and washed with methanol until the absorbance of the effluent at 238 mμ is less than 0.010; this procedure removes material which would interfere with the spectral quantification of 3-oxo-Δ^4 steroids. Subsequently the resin is washed with ethanol and then with enough water to remove all ethanol.

Table II illustrates that, when crystalline cortisone-21-glucosiduronic acid was added to a mixture of water, sulfuric acid, and sodium hydroxide and processed by use of an Amberlite XAD-2 column, it could be separated from sulfate ions and inorganic acid and, as measured by absorption at 238 mμ, approximately 96% of the conjugage was present in the first two fractions of alcoholic eluate. Removal of the alcohol gave the salt-free product which could be crystallized from

[3] The conditions described in the Experimental Section for removal of the acetyl groups from IIa–f, substances in which the glucosiduronate moiety is attached to C-21, are too mild for complete removal of the acetyl groups from 3α-hydroxy-5β-pregnanes which have the glucosiduronate group attached at C-3 (V. R. Mattox and W. D. Vrieze, unpublished data).

[4] The amides corresponding to IVa–c have been prepared by Nitta *et al.* (1964).

[5] We are indebted to Rohm & Haas Co., Philadelphia, Pa., for a generous supply of this material and a description of its adsorption properties.

the appropriate solvent. About 40 g of Amberlite XAD-2 in a column will retain 1 mmole of conjugate from an aqueous solution without significant breakthrough.

A column of Amberlite XAD-2 may be used indefinitely. Before reuse, it should be washed with several bed-volumes of alcohol to ensure that all of the adsorbed organic material has been eluted and then washed with enough water to remove all alcohol. For recovery of glucosiduronic acids from aqueous salt solutions on a preparative scale or from dilute aqueous solution, we prefer to use the Amberlite XAD-2 column instead of a cation-exchange column or the technique of adding ammonium sulfate and extracting with *n*-butyl alcohol or with ether–alcohol (3:1, v/v) (Edwards *et al.*, 1953).

TABLE II: Recovery of Cortisone-21-glucosiduronic Acid by Use of Amberlite XAD-2 Column.[a]

Fractions	pH	SO_4^{2-}	Recov (%)	
1, Water	2.4	+	0.0	
2, Water	2.3	+	0.4	
3, Water	4.7	−	1.4	
4, Ethanol		−	92.0	95.9
5, Ethanol		−	3.9	
6, Ethanol		−	0.5	
7, Ethanol		−	0.2	

[a] Column contained 10 g of resin. Compound Va (128 mg) was in a total of 20 ml of aqueous solution which contained 1.25 ml of 1.00 N NaOH and 1.27 ml of 1.00 N H_2SO_4. Fractions were 20 ml each.

Glucosiduronic acids (Va-f) have been obtained also by alkaline hydrolysis of the corresponding triacetyl derivatives (IIa–f) without isolating the methyl esters as intermediates. Since the ester functions in IIa–f are hydrolyzable under mild conditions and since the 17-hydroxy steroidal glucosiduronic acids (Va–c) are sensitive to alkali (V. R. Mattox, J. E. Goodrich, and W. Vrieze, 1968, unpublished data), drastic hydrolytic conditions have been avoided. The crude glucosiduronic acids (Va–f) were obtained from their triacetyl esters (IIa–f) in yields of 80–90%. Column chromatography of the crude acids (Va–f) gave the pure acids in an over-all yield of about 55–75% from the corresponding triacetyl esters (IIa–f).

When first prepared, Vd crystallized from water without difficulty; two subsequent preparations crystallized very slowly. However, Vd crystallized readily from 1.0 M acetate buffer (pH 4.8) (prepared from sodium acetate and acetic acid). This product contained approximately 0.5 g-atom of sodium (flame photometry) per mole of 3-oxo-Δ^4 chromophore and could be recrystallized readily from cold water without loss of sodium. It migrated chromatographically in system S5 at the same rate as Vd and was converted into Vd by acidification of an aqueous solution to pH 2.0 with sulfuric acid and passage through an Amberlite XAD-2 column. The sodium-containing derivative is formulated as the dihydrate of a 1:1 molecular compound of glucosiduronic acid Vd and the sodium salt of glucosiduronic acid Vd. Analogous acid salts have been prepared from various long-chain aliphatic acids (for example, potassium hydrogen dioleate, McBain and Stewart, 1927).

In general, the free steroidal glucosiduronic acids and the methyl esters tended to crystallize from aqueous solvents with solvent of crystallization. After drying *in vacuo* at 100° most of them were hygroscopic and gained 1 or more moles of water when exposed to the atmosphere. The analytical values on IVb, Vb, and Vc (dried at 100° *in vacuo* immediately before combustion) correspond to monohydrates; those of IVc-e correspond to hemihydrates. However, we have no direct evidence for water of solvation in the samples which were analyzed. Elce *et al.* (1967) indicated that inclusion of water of crystallization in the formulas for glucosiduronic acids and their sodium salts usually gave better agreement with analytical values than were obtained from the formulas lacking solvent of crystallization. The glucosiduronic acids of cortisone and cortisol formed relatively insoluble crystalline salts with ammonia and with barium but did not give crystalline salts with calcium, zinc, and brucine.

Each of the glucosiduronic acids (Va–f) was converted to its methyl ester by treatment with diazomethane, and each ester, in turn, was reconverted into the corresponding triacetyl ester (IIa–f) by acetylation in acetic anhydride–pyridine. Also (V. R. Mattox, J. E. Goodrich, and W. Vrieze, 1968, unpublished data) the glucosiduronic acids (Va–f) were hydrolyzed by treatment with β-glucuronidase (Ketodase) to form the corresponding free steroids (Ia–f). This series of reactions indicates that no irreversible changes in structure of the steroid nucleus occurred during the transformations.

The infrared spectra of glucosiduronic acids (Ve and f) in KBr show strong bands at 1770 cm^{-1} which are not present in the spectra of Va–d. As judged by chromatography of Ve and f in systems S5–S8 these substances contained only traces of impurities. Compounds Ve and f also had bands at 1767 cm^{-1} in Nujol. When methanolic solutions of Ve and f were esterified separately with diazomethane and the infrared spectra of the residues were taken in Nujol, there was no band in the 1770-cm^{-1} region and the spectra were identical with those of the corresponding esters (IVe and f). We interpret this to mean that the band in the 1770-cm^{-1} region is characteristic of these particular compounds (Ve and f) in the solid state in KBr and in Nujol rather than being due to contamination by one of the several functions (for example, a γ-lactone) which absorbs in the 1770-cm^{-1} region. It has been reported that the frequency of the infrared absorption band of the carbonyl group of acids is strongly dependent on the physical state of the sample and that the average value for car-

bonyl frequencies in monomeric acids is 1760 cm^{-1} (Bellamy, 1954).

Experimental Section

Analyses were by Mr. J. F. Alicino, Metuchen, N. J.; samples were redried at 100° *in vacuo* immediately before analysis. Each analytical sample was homogeneous by chromatography in two or more solvent systems. Melting points were taken on a Fisher-Johns apparatus and are reported corrected. Solutions were evaporated under reduced pressure below 40° in a rotary evaporator. Unless otherwise indicated, optical rotations were determined at 26 ± 1° (*c* 1.0). Ultraviolet absorption spectra were measured in a Beckman DU spectrophotometer. The infrared spectra in Nujol were obtained on a Model 12C Perkin-Elmer instrument; spectra obtained in KBr were taken on a double-beam instrument (Beckman IR 5A). The nuclear magnetic resonance spectra were determined by W. W. Simons of Sadtler Research Laboratories.

Systems for column chromatography (support medium, Celite-545, used as received from Johns-Manville, was impregnated with 40% its weight of stationary phase) were (A) benzene–formamide, (B) 25% cyclohexane in benzene–formamide, (C) *n*-butyl acetate–toluene–methanol–water–acetic acid (50:50:50:45:5, v/v), (D) *n*-butyl acetate–*n*-butyl alcohol–water–acetic acid (40:10:45:5, v/v), and (E) 50% cyclohexane in benzene–formamide.

Systems for paper chromatography were (S1) cyclohexane–benzene (25:75, v/v)–formamide, (S2) benzene–formamide, (S3) *n*-butyl acetate–formamide, mobile phase is butyl acetate saturated with formamide–water (1:1, v/v), (S4) *n*-butyl acetate–toluene–methanol–water–acetic acid (50:50:50:45:5, v/v), (S5) *n*-butyl acetate–*n*-butyl alcohol–water–acetic acid (80:20:90:10, v/v), (S6) *n*-butyl acetate–*n*-butyl alcohol–water–acetic acid (90:10:90:10, v/v), (S7) *n*-butyl acetate–*n*-butyl alcohol–water–acetic acid (50:50:90:10, v/v) with paper previously impregnated with 0.1 M $Ba(OAc)_2$ and dried, and (S8) *n*-butyl alcohol–water. The paper was Schleicher & Schull 2043A. For systems S1–S3 the paper was impregnated with stationary phase–acetone (3:7, v/v) and developed by the Zaffaroni technique. For systems S4–S8, the Bush technique was used. Steroids were detected by observation under 254-mμ illumination and by soda fluorescence (Simspon *et al.*, 1954).

Methyl (17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (IIa). A. FROM CORTISONE (Ia). A mixture of 3.60 g (10.0 mmoles) of cortisone and 11.0 g (40.0 mmoles) of freshly prepared (McCloskey and Coleman, 1955) dry silver carbonate in 250 ml of benzene in a 500-ml flask was boiled while being stirred (magnetic bar). After about 50 ml of solvent had been distilled, a solution of 11.3 g (30.0 mmoles) of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate (Bollenback *et al.*, 1955) in 250 ml of benzene was added dropwise. This addition took about 1 hr, and the solvent was being distilled continuously from the reaction mixture during this time. Distillation was continued for 2 hr after all of the bromo compound had been added; volume was maintained by the addition of dry benzene. The hot solution was filtered. The silver salts were digested in dilute nitric acid, and the silver bromide was collected, dried, and weighed (5.38 g, 96% of 30 mmoles). The benzene filtrate was washed with water and taken to a viscous oil.

Chromatography. A 9.5-cm diameter column was packed with 700 g of Celite 545 (impregnated with 280 ml of formamide) using benzene saturated with formamide as the mobile phase. The crude steroidal glucosiduronate was dissolved in a mixture of 10 ml of formamide and 30 ml of mobile phase. This solution was added to 25 g of Celite 545, stirred to homogeneity, and added to the column. The effluent (22.0-ml fractions) was monitored by transferring 20-μl aliquots to paper and observing under 254-mμ radiation for dark spots and also by treating the paper with alkaline tetrazolium blue (Simpson *et al.*, 1954) and drying to obtain the yellow fluorescence of the 3-oxo-Δ^4 group. The intensity of fluorescence was rated on a scale of 0–4, and these values were plotted against fraction number to locate the bands which were eluted. Small amounts of product which emerged at 1.3, 2.9, and 3.6 holdback volumes were discarded. The desired component (IIa, R_F 0.27 in system S2) emerged in fractions 212–312 (5.1 holdback volumes). These fractions were combined, washed with water, and taken to dryness. Crystals were obtained from cold ethanol: yield 3.30 g, 49%, mp 197–199°; 0.33 g, 5%, mp 196–197° dried to constant weight at 90°; lit. mp 105–107° (Wotiz *et al.*, 1959), 127–129° (Zorbach and Valiaveedan, 1964), and 193° (Nitta *et al.*, 1964). Samples recrystallized from ethanol–water usually showed a preliminary melting point of 124–126°. The material crystallized nicely from ethanol and from acetone–isooctane: $[\alpha]_D$ +87 ± 2 and +86 ± 2° ($CHCl_3$) on different preparations and M_D +590; lit. $[\alpha]_D$ +121° ($CHCl_3$) (Wotiz *et al.*, 1959), +103° ($CHCl_3$) (Zorbach and Valiaveedan, 1964) and +95° (EtOH) (Nitta *et al.*, 1964); $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 16,000); ν_{max}^{KBr} 3550, 1760, 1670, 1625, 1235, 1045, 979, 892, 863, and 787 cm^{-1}; $\delta_{(CH_3)_4Si}^{CDCl_3}$ 0.64 (C-18 methyl), 1.41 (C-19 methyl), 2.01 (two acetoxyls), 2.10 (one acetoxyl), 3.78 (one methoxyl), 3.81–5.40 (protons adjacent to oxygen), 5.72 (C-4 olefinic proton), 4.15, 4.46, 4.69, 4.99 (J = 18 cps) (C-20 methylene), 4.60, and 4.72 ppm (J = 7 cps) (axial 1-proton of glucosiduronyl moiety).

Anal. Calcd for $C_{34}H_{44}O_{14}$: C, 60.34; H, 6.55; CH_3O, 4.59; CH_3CO, 19.08. Found: C, 60.23; H, 6.15; CH_3O, 4.74; CH_3CO, 19.71.

The foregoing preparation was carried out using 1 mmole of steroid with 4 mmoles of silver carbonate and various amounts of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate. After nine holdback volumes of benzene had passed through the column, the solvent was changed to chloroform and four additional holdback volumes of solvent was collected; two bands were eluted by the chloroform, the slower one being cortisone (chromatography in systems S2 and S3 and isolation as crystals). With 2 equiv of bromoglucuronate/mmole of steroid, 10% of the initial cortisone was present and the yield of IIa was 44%; with 3 equiv of bromoglucuronate only 1.5% of the unchanged cortisone was present

and the yield of IIa was 53%. When 4 mmoles of commerically prepared silver carbonate (Fisher S-173, Fisher Scientific Co.) was used with 1 mmole of steroid and 3 mmoles of bromoglucuronate, the yield of IIa was 36%, and only 56% of the theoretical amount of silver bromide (*vs.* 93% with freshly prepared silver carbonate) was formed.

When the reaction between methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate (3 mmoles) and freshly prepared silver carbonate (4 mmoles) was carried out in the absence of steroid, essentially all of the bromoglucuronate was consumed, as indicated by formation of 93% of the theoretical amount of silver bromide.

B. FROM METHYL (11β,17-DIHYDROXY-3,20-DIOXOPREGN-4-EN-21-YL 2,3,4-TRI-*O*-ACETYL-β-D-GLUCOSID)URONATE (IIB). Bis(pyridine)chromium oxide (100 mg) was added to pyridine (5.0 ml). To this mixture at room temperature was added a solution of 100 mg of 11β-hydroxy conjugate IIb in 5.0 ml of pyridine. After 1 hr, 40 ml of water was added, the solution was extracted with chloroform, and the extract was washed with an excess of dilute HCl. Subsequently, it was washed twice with an acidic solution of sodium bisulfite, with sodium bicarbonate solution, and with water and then was taken to dryness. Crystals (76.7 mg, 77%, mp 200–202°) were obtained from ethanol. The product did not depress the melting point of IIa which was derived from Ia; the infrared spectra of the two samples of IIa in Nujol were identical.

C. FROM METHYL (17-HYDROXY-3,11,20-TRIOXOPREGN-4-EN-21-YL 2,3,4-TRI-*O*-ACETYL-β-D-GLUCOSID)URONATE 3,20-BISSEMICARBAZONE (IIIa). To a solution of 25 mg of bissemicarbazone (IIIa) in 2.0 ml of chloroform and 0.5 ml of acetic acid was added 0.5 ml of 80% aqueous pyruvic acid. The solution stood at room temperature for 17 hr and was extracted with 25 ml of chloroform. The chloroform extract was washed with cold saturated sodium bicarbonate (two 3-ml portions) and water (two 3-ml portions) and taken to dryness. The product (14.5 mg, 69%, mp 185–188°) crystallized from ethanol. It was recrystallized and identified by comparison (mixture melting point and paper chromatography in systems S1 and S2) with IIa prepared from Ia.

(Compounds IIb–f were prepared from the respective bissemicarbazones IIIb–f and identified in a similar manner.)

D. FROM METHYL (17-HYDROXY-3,11,20-TRIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONATE (IVa). A solution of 100 mg of ester IVa in 1.0 ml of pyridine and 1.0 ml of acetic anhydride was left at room temperature for 6 hr, and then ice and water were added. (A subsequent experiment showed that acetylation was essentially complete in 30 min.) The mixture was extracted with chloroform; the organic phase was washed twice with dilute HCl, with sodium bicarbonate solution, and with water and then was taken to dryness. Crystallization from ethanol gave 104 mg (constant weight at 90°) of product which melted at 198–199° and did not depress the melting point of IIa prepared from Ia. The chromatographic mobilities on paper in systems S1 and S2 were the same as those of IIa prepared from Ia.

(Compounds IIb–f were prepared from their methyl esters similarly and identified by the same general procedure.)

Methyl (11β, 17-dihydroxy-3,20-dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (IIb) was prepared from cortisol (Ib) as described for IIa from Ia. The reaction product was chromatographed in system A (holdback volume = 11). On crystallization from ethanol–water, the following was observed: yield 37%; mp 122–124°, $[\alpha]_D$ +72 ± 2° ($CHCl_3$), $\lambda_{max}^{CH_3OH}$ 242 mμ (ε 16,400); lit. mp 118–120°, $[\alpha]_D$ +69 ± 2° (EtOH), λ_{max}^{EtOH} 242 mμ (ε 17,790) (Nitta *et al.*, 1964); ν_{max}^{KBr} 3500, 1760, 1660, 1620 (s), 1220 (w), 1045 (w), 980 (s), 895, 867, and 785 (w) cm^{-1}.

Anal. Calcd for $C_{34}H_{46}O_{14}$: C, 60.16; H, 6.83; CH_3O, 4.57; CH_3CO, 19.01. Found: C, 59.86; H, 6.50; CH_3O, 4.88; CH_3CO, 19.76.

Methyl (17-hydroxy-3,20-dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (IIc) was prepared from Ic as above. The product was chromatographed in system B (holdback volume = 5.9). On crystallization from methanol–water, the following was observed: yield 36%; mp 115–117°, $[\alpha]_D$ +38 ± 2° ($CHCl_3$), $\lambda_{max}^{CH_3OH}$ 241 mμ (ε 16,700); lit. mp 134–136°, $[\alpha]_D$ +48 ± 4° (EtOH), λ_{max}^{EtOH} 242 mμ (ε 10,400) (Nitta *et al.*, 1964); ν_{max}^{KBr} 3500, 1760, 1675, 1620 (s), 1220 (w), 1040 (w), 978 (s), 888 (w), and 782 (w) cm^{-1}. (For chromatographically pure Ic, $[\alpha]_D$ +126 ± 2° (EtOH); this value is used for calculating M_D of IIc in Table I.)

Anal. Calcd for $C_{34}H_{46}O_{13}\cdot H_2O$: C, 59.99; H, 7.11. Found: C, 59.97; H, 7.09.

Methyl (11β-hydroxy-3,20-dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (IId) was made from corticosterone (Id) as described for IIa from Ia. This was chromatographed in system B (holdback volume = 3.5). On crystallization from CH_3OH, the following was observed: yield 51%; mp 168–169°; $[\alpha]_D$ +103 ± 2° ($CHCl_3$); $\lambda_{max}^{CH_3OH}$ 240 mμ (ε 16,000); ν_{max}^{KBr} 3550, 1760, 1710, 1680, 1625, 1230 (ws), 1045, 982, 898, 870, and 772 cm^{-1}.

Anal. Calcd for $C_{34}H_{46}O_{13}$: C, 61.62; H, 7.00; CH_3CO, 19.49. Found: C, 62.11; H, 6.89; CH_3CO, 18.51.

Methyl (3,11,20-Trioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (IIe). Compound IId (2 g) was oxidized with bis(pyridine)chromium oxide (see IIa from IIb), and 1.74 g (87%) of crystalline IIe was obtained from methanol: mp 166–167°; $[\alpha]_D$ +112 ± 2° ($CHCl_3$); $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 16,100); ν_{max}^{KBr} 1755, 1710 (s), 1670, 1620, 1220, 1040 (w), 977, 895, 865, and 772 cm^{-1}.

Anal. Calcd for $C_{34}H_{44}O_{13}$: C, 61.81; H, 6.71. Found: C, 61.75; H, 6.56.

Methyl (3,20-dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (IIf) was prepared from 11-deoxycorticosterone (If) by the procedure used for making IIa from Ia and chromatographed in system E; holdback volume = 2.4. On crystallization from CH_3OH, the following was observed: yield 45%; mp 205–207°; lit. mp 205–207.5° and $[\alpha]_D$ +68° (*c* 2.0, $CHCl_3$) (Zorbach, 1958), mp 199.5–201.5° (Pelzer, 1959);

ν_{max}^{KBr} 1750, 1710, 1680, 1620 (s), 1225, 1040, 990 (s), 895 (w), 875, and 787 cm^{-1}.

Methyl (17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate 3,20-Bissemicarbazone (IIIa). To a solution of 375 mg of semicarbazide hydrochloride and 252 mg of sodium bicarbonate in 1.0 ml of water was added 25 ml of methanol and 338 mg of triacetyl ester IIa. After the solution had remained at room temperature for 22 hr, 385 mg of crystals (yield 99%, mp >220° dec) was collected. The product was recrystallized by dissolving it in chloroform–methanol and concentrating the solution; $\lambda_{max}^{CH_3OH}$ 269 mμ (ε 34,000).

Anal. Calcd for $C_{36}H_{50}N_6O_{14}$: C, 54.67; H, 6.37; N, 10.63. Found: C, 54.72; H, 6.43; N, 10.66.

The following five compounds were prepared by the procedure just described.

Methyl 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate 3,20-bissemicarbazone (IIIb) was crystallized from CH_3OH. The following was observed: yield 95%, mp >220° dec, $\lambda_{max}^{CH_3OH}$ 268 mμ (ε 35,500).

Anal. Calcd for $C_{36}H_{52}N_6O_{14}\cdot H_2O$: C, 53.32; H, 6.71; N, 10.37. Found: C, 53.23; H, 6.56; N, 10.55.

Methyl (17-hydroxy-3,20-dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate 3,20-bissemicarbazone (IIIc) was crystallized from CH_3OH–H_2O. The following was observed: yield 100%, mp >210° dec, $\lambda_{max}^{CH_3OH}$ 268 mμ (ε 36,200).

Anal. Calcd for $C_{36}H_{52}N_6O_{13}$: C, 55.66; H, 6.75; N, 10.82. Found: C, 55.26; H, 6.98; N, 10.46.

Methyl (11β-hydroxy-3,20-dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate 3,20-bissemicarbazone (IIId) was crystallized from CH_3OH–H_2O. The following was observed: yield 100% mp >210° dec, $\lambda_{max}^{CH_3OH}$ 269 mμ (ε 32,700).

Anal. Calcd for $C_{36}H_{52}N_6O_{13}\cdot H_2O$: C, 54.40; H, 7.10; N, 10.57. Found: C, 54.71; H, 6.68; N, 10.44.

Methyl (3,11,20-trioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate 3,20-bissemicarbazone (IIIe) was crystallized from CH_3OH–H_2O. The following was observed: yield 100%, mp >200° dec, $\lambda_{max}^{CH_3OH}$ 269 mμ (ε 30,000).

Anal. Calcd for $C_{36}H_{50}N_6O_{13}$: C, 55.80; H, 6.50; N, 10.85. Found: C, 56.22; H, 6.62; N, 10.64.

Methyl (3,20-Dioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate 3,20-Bissemicarbazone (IIIf). A. FROM METHYL (3,20-DIOXOPREGN-4-EN-21-YL 2,3,4-TRI-*O*-ACETYL-β-D-GLUCOSID)URONATE (IIf). Compound IIf was crystallized from CH_3OH–H_2O. The following was observed: yield 97%, mp 188–189° dec, $\lambda_{max}^{CH_3OH}$ 269 mμ (ε 32,800).

Anal. Calcd for $C_{36}H_{52}N_6O_{12}$: C, 56.83; H, 6.89; N, 11.05. Found: C, 56.61; H, 7.09; N, 10.55.

B. FROM METHYL (3,20-DIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONATE 3,20-BISSEMICARBAZONE (VIf). Compound VIf (5 mg) was acetylated in a mixture of 0.5 ml of pyridine and 0.5 ml of acetic anhydride during 3 hr and the product was washed as described for IIa from IVa. Chromatography of aliquots of the residue on paper in systems S3 and S4 revealed a single spot with the mobility of IIIf. The residue gave crystals (3.3 mg, mp 188–190°, no depression on admixture with IIIf) from methanol–water.

Compound IIIa was prepared from VIa in a similar manner.

Methyl (17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (IVa) from Methyl (17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl 2,3,4-tri-O-acetyl-β-D-glucosid)uronate (IIa). A. SODIUM METHYLATE AS CATALYST. To 1.00 g of triacetyl ester IIa was added 40 ml of dry methanol containing 0.3 ml of 1.2 N sodium methylate. The mixture became homogeneous in 5 min. After 30 min, a slight excess of acetic acid was added and the solution was concentrated. Crystals (649 mg when dried to constant weight at 90°, 80%, mp 142–143.5°) were obtained from methanol. A sample, recrystallized from methanol and dried at room temperature, lost 6.8% at 1 mm and 100° for 1 hr (calculated for loss of CH_3OH, 5.64%). The product was slightly hygroscopic. When crystallized from acetone–ethyl acetate, the following was observed: mp 191–193°, $[\alpha]_D$ +93 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 16,000).

Anal. Calcd for $C_{28}H_{38}O_{11}$: C, 61.08; H, 6.96; CH_3O, 5.64. Found: C, 61.37; H, 6.96; CH_3O, 5.85.

B. SODIUM HYDROXIDE AS CATALYST. To 677 mg of triacetyl ester IIa in 10 mg of benzene was added 5.0 ml of methanol and then 5.0 ml of 0.04 N sodium hydroxide in methanol. After 15 min, a slight excess of acetic acid was added, the solution was concentrated to dryness, and crystals (504 mg dried at 100°, 91% yield, mp 140–143°) were obtained from methanol. Chromatography in system S4 showed that the product contained less than 1% of impurity.

C. FROM (17-HYDROXY-3,11,20-TR[1]OXOAREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONIC ACID (Va). To 40 mg of acid Va in 10 ml of methanol was added an excess of diazomethane in ether. After 20 min, a few drops of dilute acetic acid were added, the solution was concentrated to dryness, and the residue was crystallized from methanol. The product (18.3 mg, mp 139–143°) was identified as IVa by mixture melting point determination and comparison of infrared spectra (Nujol).

(Esters IVb–f were prepared from the corresponding acids and identified similarly. When ester IVd (which could not be obtained in crystalline form when prepared from IId) was prepared from Vd it gave crystals from ethyl acetate, mp 130–134°.)

The following five compounds were prepared from their triacetyl esters using sodium hydroxide as catalyst as described in the preparation of IVa.

Methyl (11β,17-Dihydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (IVb). On crystallization from CH_3OH–H_2O, the following was observed: yield 83%, mp 144–147°, $[\alpha]_D$ +76 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 242 mμ (ε 16,100).

Anal. Calcd for $C_{28}H_{40}O_{11}\cdot H_2O$: C, 58.93; H, 7.42; CH_3O, 5.44. Found: C, 58.90; H, 7.44; CH_3O, 5.41.

Methyl (17-hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (IVc) was chromatographed in system C; holdback volume = 3.1. When crystallized from ethyl acetate, the following was observed: yield 54%, mp 163–165°, $[\alpha]_D$ +49 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 240 mμ (ε 16,400).

Anal. Calcd for $C_{28}H_{40}O_{10}\cdot 0.5H_2O$: C, 61.63; H, 7.58. Found: C, 61.51; H, 7.24.

Methyl (11β-hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (IVd) was chromatographed in system C; holdback volume = 3.5. The yield of homogeneous product (chromatography in systems S4 and S6) when based on absorbance at 239 mμ was 77%. This product could not be crystallized (see IVd from Vd in which crystals were obtained). For homogeneous amorphous product, dried at 100° and 1 mm, the following was observed: $[\alpha]_D$ +97 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 239 mμ (ε 16,100).

Anal. Calcd for $C_{28}H_{40}O_{10}\cdot 0.5H_2O$: C, 61.63; H, 7.57; CH_3O, 5.68. Found: C, 61.25; H, 7.37; CH_3O, 5.88.

Methyl (3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (IVe) was chromatographed in system C; holdback volume = 3.0. When crystallized from ethyl acetate, the following was observed: yield 79%, mp 199–200° dec, $[\alpha]_D$ +108 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 16,100).

Anal. Calcd for $C_{28}H_{38}O_{10}\cdot 0.5H_2O$: C, 61.86; H, 7.23; CH_3O, 5.71. Found: C, 61.90; H, 7.36; CH_3O, 6.54.

Methyl (3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (IVf) was chromatographed in system C; holdback volume = 1.9. The following was observed: amorphous, $[\alpha]_D$ +75 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 241 mμ (ε 16,600).

Anal. Calcd for $C_{28}H_{40}O_9$: C, 64.59; H, 7.74; CH_3O, 5.96. Found: C, 65.01; H, 7.70; CH_3O, 6.54.

(17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosid)uronic Acid (Va). A. RECOVERY BY BUTANOL EXTRACTION. To 300 mg (0.55 mmole) of methyl (17-hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosid)uronate was added 30 ml of 0.1 N aqueous sodium hydroxide; the steroid dissolved within 5 min. After 25 min the pH was brought to 2.0 (glass electrode) by addition of 1.0 N sulfuric acid; 16.0 g of ammonium sulfate was added, and the mixture was extracted with three 35-ml portions of *n*-butyl alcohol. The combined alcoholic extracts were filtered and evaporated to dryness. The residue was dissolved in a mixture of 0.8 ml of lower phase and 2.0 ml of upper phase of system D, and the mixture was added to 2.0 g of Celite 545 and transferred to a chromatography column (100 g of Celite 545 containing 40 ml of lower phase of system D packed in a 3.6-cm diameter column). The main product appeared in fractions 38–64 (14-ml fractions) with maximal concentration in fraction 51 (3.2 holdback volumes). Fractions 38–64 were combined and concentrated to about 50 ml; 50 ml of water was added and concentration was continued. Crystals were obtained from cold water (168 mg, 58%, constant weight over $CaCl_2$). The product, which crystallized from water–acetone, from methanol–ethyl acetate, and from methanol–methyl ethyl ketone, was hygroscopic. For the anhydrous substance, the following was observed: mp 197–199° (on apparatus at 190°); $[\alpha]_D$ +94 ± 2° (CH_3OH); $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 16,200); $\lambda_{max}^{H_2O}$ 245 mμ (ε 16,400); neut equiv calculated 537 and found 539; ν_{max}^{KBr} 3420, 2940, 1755 (s), 1703, 1656, 1355, 1091, 920, and 883 cm^{-1}; $\delta_{TMS}^{(CD_3)_2SO}$ 0.50 (C-18 methyl), 1.31 (C-19 methyl), 2.90–5.40 (protons adjacent to oxygen), and 5.64 ppm (C-4 olefinic proton).

When preparation of acids Va–f from the respective esters was carried out as just described, except that 1.0 N ammonium hydroxide was used in place of sodium hydroxide, each acid contained a small amount of product, presumably the corresponding amide, which migrated chromatographically in systems S5 and S7 at the same rate as the amides described subsequently.

B. RECOVERY BY USE OF ION-EXCHANGE RESIN. A solution of 550 mg (1.00 mmole) of ester IVa in 50 ml of 0.1 N aqueous sodium hydroxide stood at room temperature for 30 min, and then an excess of acetic acid was added. The solution was poured into a column containing 10 g (12 mequiv) of 50–100 mesh Dowex 50W-X2 resin in the acid cycle and was eluted with seven 20-ml portions of water–methanol (1:1, v/v). The eluates were combined and concentrated almost to dryness. Crystals (398 mg, mp 196–199°; 76 mg, mp 195–198°; dried to constant weight at 100°) were obtained from water in 91% yield.

For use with sodium steroidal glucosiduronates in general the column, in the acid cycle, should be washed with water immediately before use; otherwise, nonvolatile acid which is formed slowly by breakdown of the resin will emerge in the effluent. Concentration of such an effluent to dryness *in vacuo* followed by an attempt to crystallize the product from methanol can lead to formation of the methyl ester of the glucosiduronic acid.

(11β,17-Dihydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronic acid (Vb) was prepared as described for acid Va, procedure A, and chromatographed in system D; holdback volume = 3.0. On crystallization from CH_3OH–EtOAc, the following was observed: yield 48%; mp 158–160°; $[\alpha]_D$ +73 ± 2° (CH_3OH); $\lambda_{max}^{CH_3OH}$ 242 mμ (ε 16,100); ν_{max}^{KBr} 3380, 2940, 1720, 1660 (b), 1350, 1234, 1058, 943, and 869 cm^{-1}.

Anal. Calcd for $C_{27}H_{38}O_{11}\cdot H_2O$: C, 58.26; H, 7.24. Found: C, 58.32; H, 6.82.

(17-Hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronic acid (Vc) was prepared as for acid Va, procedure A, except not chromatographed. When crystallized from H_2O, the following was observed: yield 76%; mp 179–181° dec; $[\alpha]_D$ +51 ± 2° (CH_3OH); $\lambda_{max}^{CH_3OH}$ 241 mμ (ε 16,900); ν_{max}^{KBr} 3415, 2940, 1725, 1645, 1368, 1235, 1087, 1033, 951, 921, 900, and 884 cm^{-1}.

Anal. Calcd for $C_{27}H_{38}O_{10}\cdot H_2O$: C, 59.98; H, 7.46. Found: C, 59.46; H, 7.27.

(11β-Hydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronic acid (Vd) was prepared as described for acid Vc from IVc. When crystallized from H_2O, the following was observed: yield 63%; mp 159–160° dec; $[\alpha]_D$ +104 ± 2° (CH_3OH); $\lambda_{max}^{CH_3OH}$ 241 mμ (ε 15,800); ν_{max}^{KBr} 3420, 2940, 1720, 1664, 1350, 1238, 1068 (br), 947, 915, and 870 cm^{-1}.

Anal. Calcd for $C_{27}H_{38}O_{10}$: C, 62.05; H, 7.33. Found: C, 62.15; H, 7.33.

(3,11,20-Trioxopregn-4-en-21-yl β-D-glucopyranosid)uronic acid (Ve) was prepared as described for acid Vc from IVc. When crystallized from CH_3OH, the following was observed: yield 37%; mp 236–238° dec; $[\alpha]_D$

+121 ± 2° (CH_3OH); $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 15,500); ν_{max}^{KBr} 3440, 3230, 2980, 1770, 1700, 1642, 1281, 1237, 1066, 1032, 962, 944, 920, and 871 cm^{-1}.

Anal. Calcd for $C_{27}H_{36}O_{10}$: C, 62.29; H, 6.97. Found: C, 61.79; H, 7.07.

(3,20-Dioxopregn-4-en-21-ylβ-D-glucopyranosid)uronic acid (Vf) was prepared as described for acid Vc from IVc. When crystallized from ethanol, the following was observed: yield 81%; mp 214–215° dec; $[\alpha]_D$ +82 ± 2° (CH_3OH); $\lambda_{max}^{CH_3OH}$ 240 mμ (ε 16,300); ν_{max}^{KBr} 3410, 3220, 2950, 1770, 1700, 1645, 1358, 1280, 1235 (w), 1220, 1163, 1128, 1075, 975, 954, 882, and 873 cm^{-1}.

Anal. Calcd for $C_{27}H_{38}O_9$: C, 64.01; H, 7.56. Found: C, 63.63; H, 7.44.

Sodium Hydrogen Bis[(11β-hydroxy-3,11-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate]. To 193 mg (0.37 mmole) of amorphous, chromatographically pure Vd in 2.0 ml of H_2O was added 50 mg (0.37 mmole) of $NaOAc \cdot 3H_2O$ in 1 ml of water. The solution was refrigerated to obtain crystals (87 mg, mp 170–174°, dried at 100°); a second crop (20 mg, mp 170–172°) separated when the solution was concentrated. The product was recrystallized from water without loss of sodium. A sample which had been dried at 100° gained 10% during exposure to the atmosphere for 2 hr. For the analytical sample, dried at 100° and 740 mm, the following was observed: mp 170–172° and $\lambda_{max}^{CH_3OH}$ 241 mμ (ε 16,400).

Anal. Calcd for $C_{27}H_{38}O_{10} \cdot C_{27}H_{37}NaO_{10} \cdot 2H_2O$: C, 58.79; H, 7.22; CH_3CO, 0.0; Na, 2.06. Found: C, 58.94; H, 7.06; CH_3CO, 0.0; Na, 1.99.

Preparation of Amberlite XAD-2 Column. Amberlite XAD-2 (40 g) was suspended in methanol and poured into a 1.8-cm diameter column. The resin was washed with methanol until the absorbance of the eluate at 238 mμ was less than 0.010. Then it was washed with ethanol until the absorbance was less than 0.010, and finally it was washed with several bed-volumes of water. At this stage it was ready for application of the aqueous solution of steroidal glucosiduronic acid. (To regenerate the column after use, it was washed with 200 ml of ethanol and then with 200 ml of water. The bubbles in the column, which formed when alcohol was added to elute the conjugate and which remained throughout the elution procedure and the subsequent washing with ethanol, were removed by filling the column almost full with water, adding a stopper, and inverting the column several times. Finally, the resin was washed with an additional 1300 ml of water.)

Recovery of Glucosiduronic Acids Using Amberlite XAD-2 Column. A. (17-HYDROXY-3,11,20-TRIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONIC ACID (Va). To 667 mg of methyl triacetyl ester IIa was added 50 ml of 0.2 N methanolic sodium hydroxide and the mixture was swirled until it became homogeneous. Five minutes later, 50 ml of water was added and the solution stood for an additional 5 min. Then, an excess of dilute acetic acid in methanol was added and the solution was taken to dryness. The residue was dissolved in 80 ml of water and the pH was brought to 2.0 (glass electrode) by addition of 1 N sulfuric acid. The solution was poured onto the Amberlite XAD-2 column and allowed to percolate through the resin under gravity at a rate of about 10 ml/min. The effluent was collected in 80-ml fractions. When all of the solvent had passed into the column, 80 ml of water was added. Subsequently, the column was washed with an additional 80-ml portion of water and with two 80-ml portions of ethanol. Fractions 1–3 (aqueous solutions) of eluate were discarded. Fractions 4 and 5 (alcoholic solutions) of eluate were combined and taken to dryness. Crystals (mp 194–195° dec) were obtained from cold water. In four preparations, the yields, dried over $CaSO_4$, were 78, 84, 92, and 92%. When chromatographed on paper in system S5 and evaluated by observation over 254-mμ radiation and by the soda fluorescence test, the principal product (Va, R_F 0.28) was contaminated with approximately 2% of material with R_F 0.75 and a trace of material with R_F 0.49. No starting material (IIa, R_F 0.93) or methyl ester (IVa, R_F 0.62) was present.

Chromatography. The combined crystals of Va (derived from 4 mmoles of IIa) were dissolved in a mixture of 6 ml of stationary phase and 15 ml of mobile phase of system D. This solution was mixed with 15 g of Celite 545 and the mixture was transferred to a 5.8-cm diameter column containing 310 g of Celite 545 which had been impregnated with 124 ml of the heavier phase of system D. The column was developed with the lighter phase of system D and aliquots of effluent were evaluated on paper by the soda fluorescence test. Small amounts of products which emerged at 1.2 and 1.6 holdback volumes were discarded. Compound Va emerged at 3.2 holdback volumes. Crystallization of this product from cold acetone gave 1.24 g (58% yield from IIa) of chromatographically pure Va, mp 226–227° dec (on apparatus at 220°).

B. (11β,17-DIHYDROXY-3,20-DIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONIC ACID (Vb). This acid was obtained from ester IIb in 80% yield (by ultraviolet absorption) as amorphous homogeneous material (system S5) by the procedure described in the foregoing two paragraphs.

C. (17-HYDROXY-3,20-DIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONIC ACID (Vc). Acid Vc was obtained from ester IIc in 77% yield; it still contained about 1% impurity.

D. (3,11,20-TRIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONIC ACID (Ve). This product was obtained from ester IIe in 64% yield; it was chromatographically pure (system S5) by crystallization from CH_3OH without prior chromatography on the Celite column.

E. (3,20-DIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONIC ACID (Vf). When the hydrolyzate of ester IIf, containing acid Vf, was to be applied to the Amberlite XAD-2 column the volume of solvent was increased to 180 ml and the pH was brought no lower than 2.9; this modification of the procedure was made in order to keep conjugate Vf in solution. Acid Vf was obtained from ester IIf in 77% yield and was chromatographically pure (system S5) without prior partition chromatography on the Celite column.

Methyl (17-hydroxy-3,11,20-trioxopregn-4-en-21-yl

β-D-glucopyranosid)uronate 3,20-bissemicarbazone (VIa) was prepared from ester IVa by the procedure used for making IIIa from IIa except that the solution was concentrated and water was added before crystals were obtained: yield 85%, mp >200° dec, $\lambda_{max}^{CH_3OH}$ 269 mμ (ε 32,600).

Anal. Calcd for $C_{30}H_{44}N_6O_{11}\cdot 0.5H_2O$: C, 53.45; H, 6.73; N, 12.48. Found: C, 53.43; H, 7.04; N, 11.95.

Methyl (3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate 3,20-bissemicarbazone (VIf) was prepared as described for VIa: yield 46%, mp 190° dec, $\lambda_{max}^{CH_3OH}$ 269 mμ (ε 32,600).

Anal. Calcd for $C_{30}H_{46}O_9N_6$: C, 56.77; H, 7.31. Found: C, 56.71; H, 7.55.

Ammonium (17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (VIIa). A. FROM METHYL (17-HYDROXY-3,11,20-TRIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONATE (IVa). To 220 mg of ester IVa was added 20 ml of 1.0 N ammonium hydroxide. After 30 min at room temperature, the solution was taken to dryness. Crystallization from water–acetone gave 185 mg of ammonium salt, mp 150° dec, which retained 2 moles of water/mole when dried at 25° and 1 mm. That the ammonium salt prepared by this procedure contained a small amount of glucosiduronamide was shown by chromatography in system S7.

B. FROM (17-HYDROXY-3,11,20-TRIOXOPREGN-4-EN-21-YL β-D-GLUCOPYRANOSID)URONIC ACID (Va). Pure ammonium salt VIIa was prepared by adding 1.5 times theory of 0.3 N ammonium hydroxide to a solution of the acid (Va) in water, concentrating immediately, and adding acetone: $[\alpha]_D$ +88 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 15,600).

Anal. Calcd for $C_{27}H_{39}NO_{11}\cdot 2H_2O$: C, 55.00; H, 7.35; N, 2.38. Found: C, 54.85; H, 7.17; N, 2.31.

Ammonium (11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (VIIb) was prepared from acid Vb and crystallized from acetone–water: yield 77%, mp >150° dec, $[\alpha]_D$ +66 ± 2° (CH_3OH), $\lambda_{max}^{CH_3OH}$ 242 mμ (ε 16,000).

Anal. Calcd for $C_{27}H_{41}NO_{11}\cdot H_2O$: C, 56.52; H, 7.55; N, 2.45. Found: C, 56.55; H, 7.37; N, 2.25.

(17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosid)uronamide (VIIg). A solution of 200 mg of ester IVa in 20 ml of methanol saturated with ammonia at 0° was kept at 0° for 18 hr and then concentrated to dryness. Crystals (157 mg) were obtained from methanol. On chromatography in system S6 on paper impregnated with 0.1 M lead acetate, four by-products, totaling about 5%, were detected. These impurities were not removed by recrystallization from methanol.

Chromatography. The product was chromatographed on a column in system D. Powdered $Pb(OAc)_2\cdot 3H_2O$ (25 g) was mixed with 350 g of Celite 545; 140 ml of lower phase and 3000 ml of upper phase was added and the mixture was packed in a 9.5-cm diameter column. The steroid was dissolved in a mixture of 20 ml of lower phase and 25 ml of upper phase and this solution was added to a mixture of 50 g of Celite 545 and 3.6 g of $Pb(OAc)_2\cdot 3H_2O$; this mixture was transferred to the column. The glucosiduronic acid amide was eluted as a band in 2.6 holdback volumes. The residue from this band was freed of Pb^{2+} by dissolving it in water and extracting the solution repeatedly with a chloroform solution of diphenylthiocarbazone. Absence of Pb^{2+} in the aqueous phase was indicated when a dilute solution of diphenylthiocarbazone (0.005% in chloroform) did not give a pink color when mixed with the aqueous solution. The amide crystallized from methanol: mp 240° dec, lit. mp 242.5–243° (Nitta *et al.*, 1964). A pure sample, dried at 100°, absorbed 1 mole of water/mole when exposed to air, $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 16,000).

Anal. Calcd for $C_{27}H_{37}NO_{10}\cdot H_2O$: C, 59.54; H, 7.03; N, 2.57. Found: C, 59.53; H, 6.67; N, 2.27.

Amides from Esters IVa–f. Amounts (1 mg) of esters IVa–f were treated separately with 1.0 ml of alcoholic ammonia overnight and the solutions were evaporated to dryness. Aliquots of each residue were chromatographed in system S5 on paper (which previously had been impregnated with 0.1 M lead acetate in water and dried in air) with samples of acids Va–f which had been prepared from the corresponding methyl esters by hydrolysis with aqueous ammonium hydroxide and acidification. After chromatography, the papers were treated with alkaline tetrazolium blue to develop the fluorescence of the 3-oxo-Δ^4 group. As judged by the intensity of fluorescence, each of the acids (Va–f) contained 2–5% of material which migrated at the same rate as that of its corresponding amide. In system S5, each glucosiduronic acid migrates at approximately the same rate as its corresponding amide, and the presence of amides is not detected by chromatography in this system. In system S5 in the presence of 0.1 M lead acetate or in an analogous system, S7, in the presence of 0.1 M lead acetate (or 0.1 M barium acetate), each acid migrates considerably slower than its amide.

Barium (17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (VIIIa). To 200 mg of Va in 2.0 ml of ethanol–water (1:1, v/v) was added 2.0 ml of 0.1 M barium acetate in 50% ethanol (procedure of Westphal, 1944) containing four drops of acetic acid–ethanol (1:10, v/v). The solution was refrigerated, and 190 mg of crystals (dried at 100°) was obtained. A recrystallized, anhydrous sample (dried at 100° and 1 mm) gained 4.17% when exposed to air overnight. In system S5, the steroid was homogeneous and migrated at the same rate as the free acid (Va); mp >230° dec, $\lambda_{max}^{CH_3OH}$ 238 mμ (ε 16,000).

Anal. Calcd for $C_{27}H_{35}Ba_{0.5}O_{11}$: C, 53.66; H, 5.85; Ba, 11.37. Found: C, 53.55; H, 5.82; Ba, 11.33.

Barium (11β,17-Dihydroxy-3,20-dioxopregn-4-en-21-yl β-D-glucopyranosid)uronate (VIIIb). This salt was prepared as described for VIIIa from Va. On crystallization from ethanol, the following was observed: yield 73%, mp >240° dec, $\lambda_{max}^{H_2O}$ 248 mμ (ε 15,800).

Anal. Calcd for $C_{27}H_{37}Ba_{0.5}O_{11}$: Ba, 11.33. Found: Ba, 11.88.

Acknowledgment

We are indebted to Dr. John J. Schneider for calling to our attention the properties of Amberlite XAD-2,

and to Dr. John W. Rosevear for discussions concerning ion-exchange resins. We also wish to thank Dr. Raymond Dodson for comments on the interpretation of nuclear magnetic resonance spectra.

References

Barker, S. A., Bourne, E. J., Stacey, M., and Whiffen, D. H. (1954), *J. Chem. Soc. Pt. 1*, 171.

Bellamy, L. J. (1954), The Infrared Spectra of Complex Molecules, New York, N. Y., Wiley, pp 142–143.

Bollenback, G. N., Long, J. W., Benjamin, D. G., and Lindquist, J. A. (1955), *J. Am. Chem. Soc. 77*, 3310.

Bradlow, H. L. (1968), *Steroids 11*, 265.

Carpenter, J. D. G., and Kellie, A. E. (1962), *Biochem. J. 84*, 303.

Conchie, J., Levvy, G. A., and Marsh, C. A. (1957), *Advan. Carbohydrate Chem. 12*, 157.

Edwards, R. W. H., Kellie, A. E., and Wade, A. P. (1953), *Mem. Soc. Endocrinol. No. 2*, 53.

Elce, J. S., Carpenter, J. G. D., and Kellie, A. E. (1967), *J. Chem. Soc. C*, 542.

Foggitt, F., and Kellie, A. E. (1964), *Biochem. J. 91*, 209.

Goldschmid, H. R., and Perlin, A. S. (1961), *Can. J. Chem. 39*, 2025.

Hardegger, E., and Spitz, D. (1949), *Helv. Chim. Acta. 32 (Pt. 2)*, 2165.

Hardegger, E., and Spitz, D. (1950), *Helv. Chim. Acta 33*, 337.

Hashimoto, Y., and Neeman, M. (1963), *J. Biol. Chem. 238*, 1273.

Heard, R. D. H., Hoffman, M. M., and Mack, G. E. (1944), *J. Biol. Chem. 155*, 607.

Huebner, C. F., Overman, R. S., and Link, K. P. (1944), *J. Biol. Chem. 155*, 615.

Klyne, W. (1950), *Biochem. J. 47*, 41 (Abstr.).

Kuizenga, M. H., and Cartland, G. F. (1939), *Endocrinology 24*, 526.

Lemieux, R. U., Kullnig, R. K., Bernstein, H. J., and Schneider, W. G. (1958), *J. Am. Chem. Soc. 80*, 6098.

Mattox, V. R., and Vrieze, W. (1967), *Fed. Proc. 26*, 425.

McBain, J. W., and Stewart, A. (1927), *J. Chem. Soc. Pt. 1*, 1392.

McCloskey, C. M., and Coleman, G. H. (1955), Organic Syntheses, Coll. Vol. III, New York, N. Y., Wiley, p 434.

Meystre, C., and Miescher, K. (1944), *Helv. Chim. Acta 27 (Pt. 1)*, 231.

Neeman, M., and Hashimoto, Y. (1962), *J. Am. Chem. Soc. 84*, 2972.

Nitta, Y., and Ide, J. (1965), *Yakugaku Zasshi 85*, 169.

Nitta, Y., Nakajima, Y., Kuranari, M., Momose, A., and Ide, J. (1961), *Yakugaku Zasshi 81*, 1160; (1962), *Chem. Abstr. 56*, 1067*g*.

Nitta, Y., Shindo, M., and Takamura, K. (1964), *Chem. Pharm. Bull. (Tokyo) 12*, 450.

Pasqualini, J. R. (1963), *Bull. Soc. Chim. Biol. 45*, 277.

Pelzer, H. (1959), *Z. Physiol. Chem. 314*, 234.

Reichstein, T. (1937), *Helv. Chim. Acta 20*, 953.

Schneider, J. J., and Lewbart, M. L. (1959), *Recent Progr. Hormone Res. 15*, 201.

Schneider, J. J., Lewbart, M. L., Levitan, P., and Leiberman, S. (1955), *J. Am. Chem. Soc. 77*, 4184.

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

Simpson, S. A., Tait, J. F., Wettstein, A., Neher, R., von Euw, J., Schindler, O., and Reichstein, T. (1954), *Helv. Chim. Acta 37*, 1163.

Stacey, M., Moore, R. H., Barker, S. A., Weigel, H., Bourne, E. J., and Whiffen, D. H. (1958), *Proc. U. N. 2nd Intern. Conf. Peaceful Uses At. Energy, Geneva, 20*, 251.

Steiger, M., and Reichstein, T. (1937), *Helv. Chim. Acta 20*, 1164.

Westphal, U. (1944), *Z. Physiol. Chem. 281*, 14.

Wintersteiner, O., and Pfiffner, J. J. (1936), *J. Biol. Chem. 116*, 291.

Wotiz, H. H., Smakula, E., Lichtin, N. N., and Leftin, J. H. (1959), *J. Am. Chem. Soc. 81*, 1704.

Zorbach, W. W. (1958), *J. Org. Chem. 23*, 1797.

Zorbach, W. W., and Valiaveedan, G. D. (1964), *J. Org. Chem. 29*, 2462.