Oxidative Fission of Pregnanediol Glucuronide

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Reactions likely to occur between urinary steroid conjugates and sodium bismuthate may be divided into two groups: (i) oxidative fission of 1:2-glycols, 1:2-ketols and α -hydroxyacids incorporated in the steroid carbon skeleton, and (ii) analogous reactions in the carbohydrate moiety of steroid glucuronides (or glucosiduronic acids*). Of the former reactions those concerning the corticosteroid side chain have been described in recent communications from this laboratory (Brooks & Norymberski, 1952, 1953; Norymberski, 1952; Norymberski, Stubbs & West, 1953). We now wish to report the oxidative cleavage

3 α -yl glucopyranosiduronate (pregnanolone glucuronide) (IIa) (Sutherland & Marrian, 1947)—was treated with sodium bismuthate in aqueous acetic acid. The crude product was chromatographed on alumina: the four fractions which were further investigated gave, in order of elution, an oil which on prolonged contact with alumina yielded 3 α -hydroxypregnan-20-one (IIc), 3 α -formoxypregnan-20 α -ol (Ib), 3 α -hydroxypregnan-20-one (IIc) and pregnane-3 α :20 α -diol (Ic). The first fraction is considered to consist of impure 3 α -formoxypregnan-20-one (IIb) on the basis of its ready elution from

by bismuthate of steroid glucosiduronic acids under conditions identical with those employed for the oxidation of corticosteroids. Part of this work has been briefly reported elsewhere (Norymberski, 1953).

Sodium pregnanediol glucuronide of pregnancy urine (Venning, 1937, 1938)—a mixture consisting mainly of sodium 20 α -hydroxypregnan-3 α -yl glucopyranosiduronate (Ia) and sodium 20-oxopregnan-

alumina and its conversion into IIc. 3 α -Formoxypregnan-20 α -ol (Ib) was characterized by its deformylation on alumina to the diol Ic and by conversion into 3 α -hydroxypregnan-20-one (IIc) on oxidation with chromic acid followed by hydrolysis on alumina. Deformylation on alumina has been described by Koechlin, Kritchevsky & Gallagher (1950) for 3 α -formoxy-5 β -steroids and by Levin, McIntosh, Spero, Rayman & Meinzer (1948) for 5:6-unsaturated 3 β -formoxysteroids, whilst Steinegger & Katz (1947) found that 3 β -formoxycholestane (5 α) is unchanged by alumina.

The oxidative cleavage of a urinary glucosiduronic acid *in situ* was next investigated. Goldfine & Cohen (1953) have shown, by means of enzymic hydrolysis with β -glucuronidase, that pregnane-3 α :20 α -diol (Ic) is excreted exclusively in

* Nomenclature as recommended by the Carbohydrate Nomenclature Sub-Committee of the Chemical Society (*J. chem. Soc.*, 1952, p. 5117): the term 'glucosiduronic acid' replaces 'glucuronide' for a glycoside in which the terminal CH_2OH has been oxidized to a carboxyl group. In the present paper 'pregnanediol glucuronide' and 'sodium pregnanediol glucuronide' are considered trivial names and are retained unchanged.

Urine (Quarter of a 24-hr. specimen)	A Treated with NaBiO ₃	B Hydrolysed with acid	C Untreated
↓ Ethyl acetate			
Crude extract	940 mg., dark gum	480 mg., dark gum	710 mg., dark gum
↓ Alkali			
Neutral extract	55 mg., partly crystalline	30 mg., partly crystalline	14 mg., gum
↓ Chromatography			
→ Fraction 1	21 mg., gum	12 mg., gum	9 mg., gum
Fraction 2	15 mg., cryst.	12 mg., cryst.	2 mg., gum
↓ High vacuum 190°			
Sublimate	11 mg., oil and crystals*	9.7 mg., oil and crystals	Trace
↓ Recryst. from acetone			
Pure pregnane- 3α:20α-diol	5.3 mg.	4.6 mg.	—

* At this stage an unidentified by-product was separated (see p. 878).

Fig. 1. Isolation of pregnane-3α:20α-diol from pregnancy urine.

conjugation with glucuronic acid. The quantity of the diol (Ic) isolated from pregnancy urine after treatment with sodium bismuthate as compared with that obtained after acid hydrolysis should therefore indicate the efficacy of the oxidative procedure. The comparative experiments were carried out as shown in Fig. 1: oxidative or hydrolytic treatment gave pregnanediol (Ic) in approximately the same yield. To ensure that no free diol was present in the urine specimen under investigation, the extraction of an untreated sample was carried out as well.

EXPERIMENTAL

Melting points were taken on a Kofler stage. Specific rotations were determined in ethanolic solutions (c, 0.5–1) at 15–20°. Al₂O₃ (Hopkin & Williams) was treated as previously described (Brooks & Norymberski, 1953). Microanalyses are by Weiler and Strauss, Oxford; samples for analyses were prepared by sublimation in a high vacuum, unless otherwise stated.

Oxidation of pregnanediol glucuronide with sodium bismuthate

Crude sodium pregnanediol glucuronide of m.p. 255–260° was obtained from pregnancy urine essentially by the method of Venning (1937, 1938) (cf. Haslewood, 1950). This product (310 mg.) was dissolved in aqueous acetic acid (50%, v/v, 75 ml.) and shaken with NaBiO₃ (A.R., 7.5 g.) for 30 min. at room temperature; water (75 ml.) and ethyl acetate (150 ml.) were added and the shaking was continued for 10 min. The excess of reagent was filtered off

with suction, the two layers of the filtrate were separated and the aqueous phase was extracted with ethyl acetate (4 × 100 ml.). The extract was washed successively with water (50 ml.), saturated NaHCO₃ solution (7 × 50 ml.), and water (3 × 50 ml.); it was dried with anhydrous Na₂SO₄ and evaporated to dryness. The crude product (pale-yellow gum, 175 mg.) was dissolved in benzene:ethyl acetate (99:1) and chromatographed on Al₂O₃ (5 g.) as shown in Table 1.

Fraction 1–3. 3α-Formoxypregnan-20-one (IIb). Attempts to crystallize this fraction were unsuccessful and isolation of the pure formate (IIb) was abandoned. The material was dissolved in a little light petroleum and put on a column of Al₂O₃ (1 g.). The next day the column was eluted successively with light petroleum (10 ml.), benzene (10 ml.) and benzene:ethyl acetate (90:10, 10 ml.). The first two eluates on evaporation yielded gummy residues. The last fraction gave crystals (12 mg.) which were recrystallized from light petroleum as needles (9 mg.), m.p. 143–146°, not depressed on admixture with authentic 3α-hydroxypregnan-20-one. [α]_D +106°. (Found: C, 79.7; H, 10.5. Calc. for C₂₁H₃₄O₂: C, 79.2; H, 10.8%.)

Fraction 4–8. 3α-Formoxypregnan-20α-ol (Ib). This fraction was recrystallized twice from light petroleum to give thick prisms (15 mg.) of the 3α-formoxy compound, m.p. 114–116° (with change to needles at 106°); [α]_D +56°. (For analysis, a sample was dried in a high vacuum at 100°. Found: C, 75.6; H, 10.3. C₂₃H₃₆O₂ requires: C, 75.8; H, 10.4%.)

A sample (5.2 mg.) of the pure formate (Ib) was hydrolysed on Al₂O₃ as described for 3α-formoxypregnan-20-one (IIb). Benzene:ethanol (96:4) eluted pure pregnane-3α:20α-diol (4.9 mg.), m.p. 237–239°, undepressed by authentic material. [α]_D +25°. (Found: H, 11.5. Calc. for C₂₁H₃₆O₂: H, 11.3%. Owing to breakage of the CO₂ absorption tube no value for carbon is available.)

Table 1. *Chromatography of product from bismuthate oxidation of pregnanediol glucuronide*

Fraction no.	Eluent	Volume (ml.)	Eluate	
			Weight (mg.)	M.p.
1-3	Benzene:ethyl acetate: (99:1)	18	25	—
4-8	(99:1)	30	33	80-107
9	(99:1)	6	6	—
10-14	(95:5)	30	12	131-147
15-22	(95:5 and 90:10)	50	5	90-140
23-25	(90:10)	18	1	185-195
26-38	(80:20 and 50:50)	80	33	200-235
39-41	Ethyl acetate	18	6	130-180
42-45	—	24	2	—
46-48	Ethyl acetate:ethanol: (98:2)	18	2	175-185
49-53	(98:2)	30	6	—
54-55	(96:4)	100	21	—
56-57	(80:20)	200	23	—

Oxidation of the formate Ib (17.5 mg., obtained by evaporation of the mother liquors) with CrO_3 (3.75 mg., 1.1 molar equivalents) in glacial acetic acid (2 ml.) at room temperature yielded, after the usual working-up, a gummy neutral product (17.2 mg.) which failed to crystallize. It was hydrolysed on Al_2O_3 as described above; benzene:ethyl acetate (90:10) eluted a crystalline fraction (13 mg.) which, recrystallized from light petroleum, gave needles, m.p. 145-147°, identical (mixed m.p.) with authentic 3 α -hydroxypregnan-20-one; $[\alpha]_D + 108^\circ$. (Found: C, 78.8; H, 10.8. Calc. for $\text{C}_{21}\text{H}_{34}\text{O}_2$: C, 79.2; H, 10.8%.)

Fraction 10-14. 3 α -Hydroxypregnan-20-one (IIc). Recrystallization of this fraction from light petroleum yielded needles (10 mg.), m.p. 144-146°, not depressed on admixture with authentic 3 α -hydroxypregnan-20-one, $[\alpha]_D + 105^\circ$. (Found: C, 78.5; H, 10.6. Calc. for $\text{C}_{21}\text{H}_{34}\text{O}_2$: C, 79.2; H, 10.8%.)

Fraction 26-38. Pregnane-3 α :20 α -diol (Ic). This fraction was recrystallized from acetone to give plates (27 mg.), m.p. 236-237°, undepressed by authentic pregnane-3 α :20 α -diol, $[\alpha]_D + 31^\circ$. (Found: C, 78.4; H, 11.1. Calc. for $\text{C}_{21}\text{H}_{36}\text{O}_2$: C, 78.7; H, 11.3%.)

Isolation of pregnane-3 α :20 α -diol from pregnancy urine

A 24 hr. urine specimen, obtained from a pregnant woman (38th week), was made up to 2.0 l. with distilled water. Three samples of 500 ml. were taken for the isolation of pregnane-3 α :20 α -diol by the following methods (A, B and C).

A. From urine treated with sodium bismuthate. Glacial acetic acid (500 ml.) and NaBiO_3 (A.R., 150 g.) were added to a sample (500 ml.) of the diluted urine and the mixture was shaken for 30 min. at room temperature. The excess of reagent was filtered off with suction and washed with methanol (50 ml.). A few ml. of a dilute NaHSO_3 solution were added to the filtrate to remove a slight suspension of bismuthate; the clear solution was diluted with distilled water (1.0 l.) and saturated with ethyl acetate (400 ml.). The mixture was extracted with ethyl acetate in a cascade extractor (Kies & Davis, 1951) consisting of four units, each unit containing 400 ml. of the solvent. The extract was washed with water (500 ml.) and then evaporated to dryness, yielding a dark, gummy residue (940 mg.). The crude extract was hydrolysed by boiling for 10 min. with 10% ethanolic (90%, v/v) NaOH (12 ml.); the mixture was diluted with

water (100 ml.) and extracted with CHCl_3 (5 $\times$ 20 ml.). The partly crystalline, neutral product (55 mg.) was separated into three fractions by chromatography on Al_2O_3 (1.0 g.): benzene:ether (90:10, 60 ml.) eluted gummy material (21 mg.), benzene:ethanol (95:5, 30 ml.) gave yellowish crystals (15 mg.) and benzene:ethanol (90:10, 30 ml.) eluted traces of amorphous material. The second fraction was sublimed in a high vacuum at 190° to give a colourless sublimate (11 mg.) and a brown, gummy residue. The sublimate consisted of two fractions, one formed immediately above the heating block, the other about 2 cm. higher; the two fractions were separated by cutting the sublimation tube. The lower fraction yielded, on recrystallization from acetone, plates (5.3 mg.), m.p. 235-238°, undepressed by authentic pregnane-3 α :20 α -diol, $[\alpha]_D + 30^\circ$. (Found: C, 78.5; H, 10.9. Calc. for $\text{C}_{21}\text{H}_{36}\text{O}_2$: C, 78.7; H, 11.3%.)

The upper sublimate was recrystallized from light petroleum and subsequently sublimed in a high vacuum at 80-90° to give feathery needles (1.2 mg.), m.p. 92-112°, λ_{max} 210 and 223 μ , $E_{1\%}^{1\text{cm}}$ 447 and 467. The product gave no colour with tetranitromethane nor with the Zimmermann reagent.

B. From acid-hydrolysed urine. The diluted urine sample (500 ml.) was brought to the boil, 10N-HCl (50 ml.) was added and boiling continued for 10 min. The mixture was cooled and glacial acetic acid (400 ml.), water (1.0 l.), sodium acetate (50 g.), methanol (50 ml.) and ethyl acetate (400 ml.) were added. Extraction, alkaline hydrolysis of the crude extract (480 mg.) and chromatography of the neutral product (30 mg., partly crystalline) were carried out as described under A. The first chromatographic fraction (12 mg.) consisted of a yellow gum; the second fraction (12 mg.) was crystalline and, on heating to 190° in a high vacuum, gave a colourless, partly oily sublimate (9.7 mg.); the volatile by-product described under A was not observed. The sublimate when crystallized from acetone gave plates (4.6 mg.) of pregnane-3 α :20 α -diol, m.p. 234-235°, $[\alpha]_D + 30^\circ$. (Found: C, 78.8; H, 11.4. Calc. for $\text{C}_{21}\text{H}_{36}\text{O}_2$: C, 78.7; H, 11.3%.)

C. From untreated urine. Glacial acetic acid (500 ml.), water (1.0 l.), methanol (50 ml.) and ethyl acetate (400 ml.) were added to the diluted urine sample (500 ml.). The mixture was extracted, the crude extract (710 mg.) hydrolysed and the neutral product (14 mg. yellow gum) chromatographed as described under A. The first chromatographic fraction was a yellow gum (9 mg.); the second fraction was also gummy and gave only traces of an amorphous sublimate on heating in a high vacuum.

DISCUSSION

Oxidative fission of glycosides by sodium bismuthate has not been previously described; analogous reactions with periodate are, however, well known. Huebner, Lohmar, Dimler, Moore & Link (1945) have oxidized bornyl glucopyranosiduronic acid (III) with periodate and obtained bornyl formate (VIII) in 36% yield. The mechanism of this reaction has been postulated by Huebner *et al.* (1945) (see also Halsall, Hirst & Jones, 1947) as follows: glycol fission of bornyl glucopyranosiduronic acid (III) is followed by hydroxylation of the

affect the application of the reaction to the cleavage of urinary glucosiduronic acids *in situ*, since either group of products will be extractable from urine. The quantitative recovery of pregnane-3 α :20 α -diol from bismuthate-treated urine suggests the use of this procedure as an alternative to the enzymic hydrolysis with β -glucuronidase. The concurrent degradation of corticosteroids by bismuthate, while imposing an obvious limitation on the applicability of the method, can be used to advantage as for instance in the differential estimation of glucosiduronic acids and sulphates of 'total 17-keto-steroids' (Norymberski, 1953).

dialdehyde IV to give the hydroxyaldehyde V; V is oxidized to the oxalate VI which gives glyoxal hemiacetal (VII) on hydrolysis; VII is finally oxidized to bornyl formate (VIII). Since the hypothetical intermediates V and VII have hemiacetal structures, it is conceivable that hydrolysis occurs in competition with their oxidation, in which case glyoxal bornyl hemiacetal (VII) would give borneol as well as bornyl formate. The experimental evidence of Huebner *et al.* (1945) does not exclude this possibility. The above consideration applies also to the bismuthate oxidation of pregnanediol glucuronide: whatever the actual mechanism of this reaction, the isolation of the formates Ib and IIb is best explained by the oxidative cleavage of initially formed glyoxal hemiacetals; their hydrolysis would equally well explain the formation of the free alcohols Ic and IIc. However, the last two compounds may have been formed by hydrolysis of their formates during the working-up or during chromatography on alumina, though in both operations care was taken to avoid deformylation.

Whether oxidation of glucosiduronic acids with sodium bismuthate produces formates exclusively or whether alcohols are formed as well does not

In the preceding paragraphs only the oxidative fission of glucopyranosiduronic acids has been considered, the pyranose form being the preferred structure of naturally occurring glucosiduronic acids. The degradation of a glucofuranosiduronic acid,

may be expected to proceed differently with periodate and with bismuthate, since only the latter reagent can cleave α -hydroxyacids (Rigby, 1950).

SUMMARY

1. 'Pregnanediol glucuronide' from human pregnancy urine was oxidized with sodium bismuthate to give 3 α -hydroxypregnan-20-one, pregnane-3 α :20 α -diol and their 3 α -formates.

2. Pregnan-3 α :20 α -diol was extracted from pregnancy urine treated with sodium bismuthate in approximately the same yield as from acid-hydrolysed urine.

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Active Transport of Water, Sodium, Potassium and α -Oxoglutarate by Kidney-cortex Slices

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Previous experiments from this Department (Terner, Eggleston & Krebs, 1950; Krebs, Eggleston & Terner, 1951) and by Mudge (1951*a, b*) and Aebi (1953) have shown that slices of some animal tissues suspended in a saline medium may lose or gain potassium ions according to the conditions. The present work with kidney-cortex slices is a study of the movements of sodium ions and α -oxoglutarate ions (added as substrate) and of water which accompany the movements of potassium. Part of this work has been communicated to the Biochemical Society (Whittam & Davies, 1953).

EXPERIMENTAL

Material and procedure. As it was necessary to measure the fresh weight of tissue slices before incubation, contact between tissue and medium during the slicing had to be avoided. Guinea-pigs were stunned and bled from the carotids. The kidneys were at once removed, cut longitudinally and the cortex was sliced with a dry razor according to the method of Deutsch (1936). Slices, 0.3–0.4 mm. thick, were spread on hardened filter paper (Whatman no. 541) and rapidly blotted to remove blood, urine and cell debris. The outer slices were usually rejected; samples (100–200 mg.) of the inner slices were then weighed on a torsion balance and put into 2.0 ml. of medium in 15 ml. stoppered conical cups. In anaerobic experiments, a stick of yellow phosphorus was placed in the centre well. The cups were shaken at 130 oscillations/min. (amplitude 2 cm.) at

37° in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Company, Chicago, U.S.A.), or at 0° in a mechanical rocker. After incubation the slices were removed, rapidly blotted and reweighed.

Media. Phosphate salines were used when media, gassed with O_2 , were required at various pH values, which were checked with a glass electrode before and after incubation. They were similar to medium III of Krebs (1950*b*), without the addition of the organic substrates. In all other experiments, bicarbonate saline (Krebs & Henseleit, 1932) gassed with 5% CO_2 and either 95% O_2 or 95% N_2 at 37°, or 2.8% CO_2 and either 97.2% O_2 or 97.2% N_2 at 0° was used. The pH at both 37 and 0° was thus 7.4. The media used contained 6mm-K, 155–160mm-Na and usually 10mm α -oxoglutarate as substrate. A hypotonic (approx. 0.2 osm) medium was made by diluting 2 vol. of the medium with 1 vol. of water. (The osmolar concentration (osm) is obtained by adding together the molar concentrations of all the ions present, cf. Robinson, 1950.)

Water content of tissue. The tissue water was taken to be equal to the loss of weight on drying the tissue overnight at 105°.

Determination of α -oxoglutarate. The slices, or 1 ml. samples of the media, were each placed in 10 ml. centrifuge tubes containing 2.0 ml. of 10% (w/v) trichloroacetic acid (TCA). After breaking up the tissue with a glass rod the tubes were centrifuged. Samples of the supernatants were removed (0.1 ml. from the media, 1.0 ml. from the tissue extracts) and the volume made to 4.0 ml. with TCA. The tubes were then placed for 5 min. in a boiling-water bath to decompose any acetoacetic or oxaloacetic acids present (Markees, 1951; Lichtenbell & Florijn, 1952; Kogut & Podoski, 1953).