Steroids of Pregnant Mares' Urine

4. FRACTIONATION OF THE NEUTRAL STEROIDS. EXAMINATION OF SOME NON-KETONIC FRACTIONS

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Previous papers in this series (Klyne, Schachter & Marrian, 1948; Klyne, 1948; Paterson & Klyne, 1948) have described the isolation from pregnant mares' urine of 5α-pregn-16-en-3β-ol-20-one, uranediol and 5α-pregnane-3β-ol-20-one as their sulphuric acid esters. This paper describes the fractionation of the neutral steroids of pregnant mares' urine and the detailed examination of some non-ketonic fractions. Most previous workers have concentrated their attention on the ketonic fractions (see, for example, Heard & McKay, 1939; Heard & Hofmann, 1940, 1941; Oppenauer, 1941; Prelog & Führer, 1945; the last-mentioned paper gives full references to earlier work). Marker and his colleagues in the years 1937–9 (see references on p. 707) dealt extensively with both ketonic and non-ketonic fractions, but their work was carried out with commercial urine extracts which had been so harshly treated that some of the compounds isolated may have been artifacts. Bauld & Heard (1940) have carried out a brief examination of the non-ketonic steroid fraction.

Preliminary accounts of parts of the present work have already been given (Klyne & Paterson, 1948; Brooks, Klyne & Miller, 1951*a*). The identification of uranediol as a *D*-homosteroid has been described elsewhere (Klyne, 1950).

Nomenclature follows the rules proposed by a conference at the CIBA Foundation, London, and published in *J. chem. Soc.* (1951), p. 3526. Steroid rings are distinguished as *A*, *B*, *C* and *D* (italic capital letters) and not A, B, C and D (Roman small capitals) as shown in these rules. Small capitals are undesirable since D (and L) are used for another purpose in designating configurations relative to glyceraldehyde or serine (cf. *Biochem. J.* 1948, **42**, 1). Prof. T. Reichstein draws attention to this point in the German version of the steroid nomenclature rules (*Helv. chim. Acta*, 1951, **34**, 1680).

Separation of the major steroid fractions

The extraction of the steroid conjugates from pregnant mares' urine and their fractionation have been described by Klyne *et al.* (1948). The material used for the present study consisted of the conjugates in the 'water-insoluble' and 'water-soluble' fractions which could not be purified as sulphates. These conjugates were hydrolysed to the free steroids by hydrolysis with N-hydrochloric acid at 100°, and the free steroids were separated into phenolic and non-phenolic fractions by extraction of their ether and benzene solutions with sodium hydroxide. The major separations are outlined in Fig. 1. In the course of these separations on one large batch of extracts, a small neutral fraction insoluble in benzene was removed. This proved to consist largely of trihydroxysteroids and was called the 'Triol Fraction'.

The non-phenolic material was separated into ketonic and non-ketonic fractions by the reagent T of Girard & Sandulesco (1936), and these fractions again into digitonin-precipitable (β) and digitonin-non-precipitable (α) fractions. For one batch the digitonin precipitation was carried out in 90% (v/v) methanol according to Shoppee (1946); for another batch it was carried out in 95% (v/v) ethanol at room temperature followed by precipitation of the more soluble digitonides with ether as suggested by Marker, Rohrmann & Wittle (1938). The latter method proved a simple way of obtaining uranediol from the urine extracts.

The non-ketonic β fractions (β(*M*), β1(*E*) and β2(*E*) in Fig. 1) were then submitted to chromatography on alumina as free alcohols, as acetates and as benzoates.

The non-ketonic β fractions

Separation by digitonin in methanol

The non-phenolic, non-ketonic fraction from 530 l. urine (2·39 g.) was submitted to separation by digitonin in methanol following Shoppee (1946), and the β(*M*) fraction was chromatographed over alumina. Benzene eluted a little material which apparently consisted of monohydroxy compounds; an imperfectly characterized compound *MV*, m.p. 61–63°, was obtained from this fraction. Most of the material was eluted with benzene-ether mixtures. This fraction (*B*) was acetylated and the acetates

were chromatographed over alumina. Three main groups of fractions (*BA*, *BB*, *BC*) were eluted with hexane-benzene mixtures. The second group (*BB*) yielded after tedious fractional crystallizations and chromatography small quantities of uranediol diacetate, and of a new acetate called provisionally unsaturated to tetranitromethane and shows absorption in the ultraviolet at about 200 mμ., which is compatible with the presence of an olefinic linkage (cf. Bladon, Henbest & Wood, 1951; Halsall, 1951). Its melting point is higher than that of any known isomer of pregnane- or pregnene-diol diacetate.

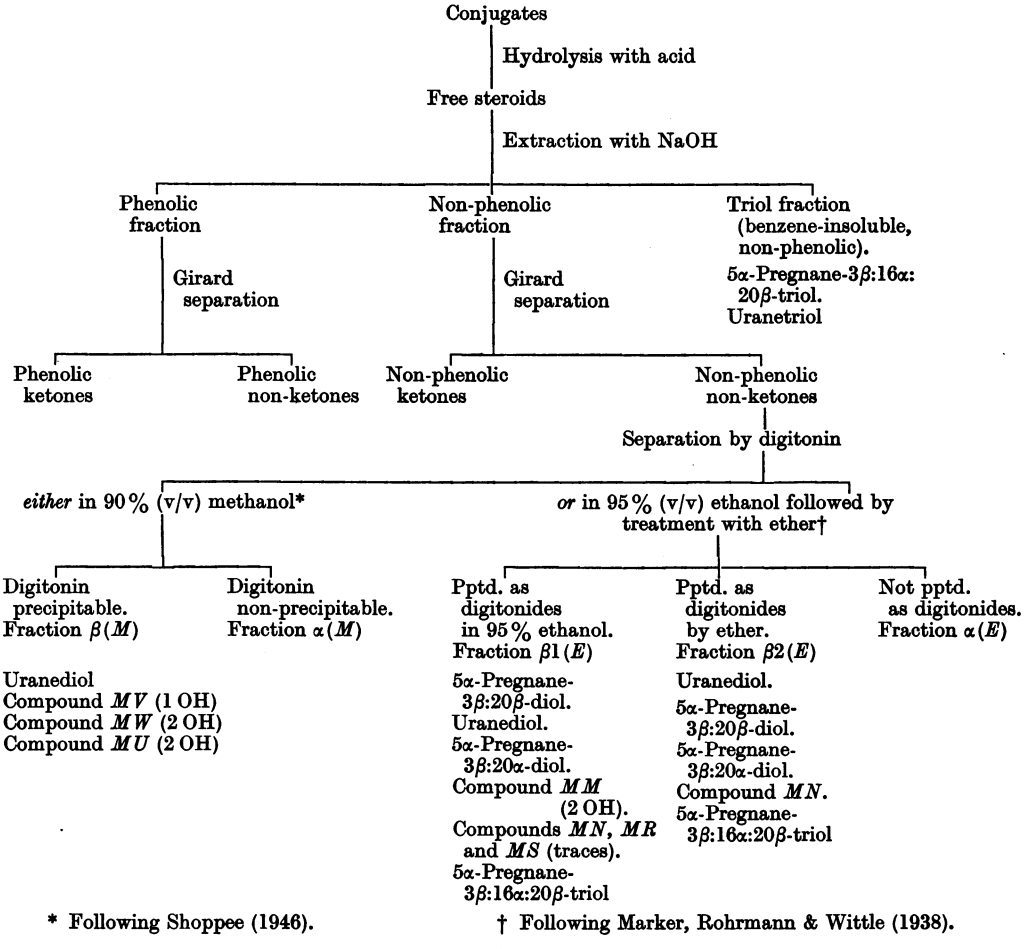


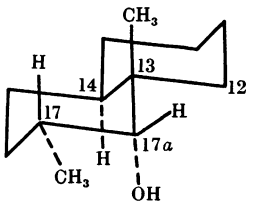
Fig. 1. Separation of the major fractions from mares' urine extracts. The compounds so far isolated in this investigation are listed under the various fractions. The only other non-phenolic, non-ketonic 3β-hydroxysteroid isolated from pregnant mares' urine is pregn-5-ene-3β:20α-diol (Marker & Rohrmann, 1938). Marker & Rohrmann (1939) obtained evidence of the presence of a urenetriol and a new 5α-pregnanetriol.

compound '*MU* diacetate', m.p. 206–208°. The many side-fractions obtained in this process were later pooled and treated by the digitonin process of Marker (see p. 696). The third group of fractions (*BC*) after repeated crystallizations yielded a further new compound '*MW* diacetate', m.p. 175·5–178°.

Compound MU. The analysis of the acetate of this compound and its behaviour on chromatography suggest that it is a diacetate $C_{25}H_{38-40}O_4$; it is

Compound MW. The analysis and chromatographic behaviour of *MW* acetate suggest the formula $C_{25}H_{40}O_4$; it is saturated to tetranitromethane. Vigorous hydrolysis of the diacetate with potassium hydroxide in methanol yielded uranediol: but *MW* diacetate ($[M]_D$, −218) could not be a polymorphic modification of uranediol diacetate ($[M]_D$, −120). Lack of material has prevented a further study of *MW* diacetate, but it seems possible that compound *MW* may be the 17*a*-epimer of

uranediol, in which the hydroxyl group at position 17*a* has the less stable 'polar' 17*a*α-position (as in I). On vigorous treatment with alkali this might perhaps rearrange to give the more stable 'equatorial' 17*a*β-hydroxyl group (as in II). (See the general discussion of 'polar' and 'equatorial' bonds



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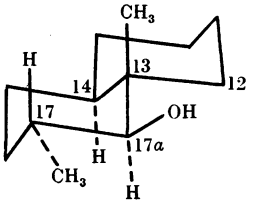


II

in steroids by Barton, 1950, where many examples of the transformation of 'polar' to 'equatorial' hydroxyl groups under the influence of alkali are described.)

Evidence for 17aβ configuration of uranediol

The formulation of uranediol as a 17α-methyl-*D*-homoandrostane-3β:17*a*-diol has been discussed by one of us previously (Klyne, 1950). The molecular rotation of uranediol dibenzoate, which has now been determined, shows that the 17*a*-hydroxyl group in this compound is β-oriented. The indices α and β at $C_{(17a)}$ are allotted here in their modern significance according to the proposals made by Goldberg, Sicé, Robert & Plattner (1947) on the basis of the rates of hydrolysis of the 17*a* esters and the androgenic activity of the alcohols. Goldberg *et al.* in their paper of 1947 actually use the 'old' indices ('α' for the androgenically active compounds which are now referred to as β-compounds).

The following are the calculations supporting the conclusion that uranediol is a 17*a*β-compound.

$[M]_D$ Uranediol dibenzoate (+118)
$-[M]_D$ uranediol (+13)
= increment on benzoylation
(Δ_2 value at $C_{(3)}$ and $C_{(17a)}$)
$= +105.$

The Δ_2 value at $C_{(3)}$ for a 3β-hydroxy-5α-steroid (Barton & Cox, 1948) is +5. Therefore Δ_2 value at $C_{(17a)} = +100$ for uranediol.

For 17*a*-epimeric alcohols Goldberg *et al.* (1947) found the following Δ_2 values: at 17*a*α, −152; 17*a*β, +227. Exact agreement between these Δ_2 values and that for 17*a* in uranediol dibenzoate cannot be expected because of the possibility of vicinal action between the two benzoate groups, and between the $C_{(17a)}$ benzoate group and the $C_{(17)}$ methyl. However, the positive sign of the Δ_2 value in uranediol dibenzoate indicates that it most probably has the 17*a*β-configuration.

The compounds *MM*, *MV* and *MW* were named *M*, *V* and *W* in our preliminary communications (Klyne & Paterson, 1948; Brooks *et al.* 1951*a*). They have been renamed with the prefix *M* to avoid confusion with the adrenal steroids of Reichstein.

Separation by digitonin in ethanol and ether

The non-ketonic material from 270 l. urine (5·18 g.) treated according to Marker, Rohrmann & Wittle (1938) yielded the following three fractions: β1(*E*), compounds precipitated by digitonin in 95% (v/v) ethanol at 15–20° (1·03 g.); β2(*E*), compounds not precipitated by digitonin in 95% ethanol, but precipitated in ether (380 mg.); α(*E*), compounds not precipitated by digitonin (3·5 g.).

Fraction β1(*E*). This material was chromatographed as free alcohols on alumina, yielding three main fractions eluted with benzene-ether mixtures, I*A*, I*B*, and I*C* (total about 800 mg.); the less easily eluted fractions (about 200 mg.) have not been studied in detail. Each of the three main fractions was again chromatographed; I*A* gave a gum, very easily eluted with benzene containing 10% ether, and then some impure uranediol: I*B* gave more impure uranediol and 5α-pregnane-3β:20β-diol (cf. Bauld & Heard, 1940): I*C* (410 mg.) gave much material which after crystallization from ethyl acetate-light petroleum appeared to be fairly pure 5α-pregnane-3β:20β-diol (170 mg.). Data proving the identity of this compound (m.p. 194·5–196°) with authentic material are given in Table 2 (p. 702).

Chromatography of benzoates. The finding of 5α-pregnane-3β:20β-diol in such large quantities led to a search for the 20α-epimer, which had been isolated from pregnant mares' urine by Marker & Rohrmann (1938). It was known from previous synthetic work (cf. Klyne & Barton, 1949) that 20-epimeric hydroxysteroids could not be readily separated by chromatography of their acetates. It was, therefore, proposed to try the use of the benzoates, which had been employed successfully by Barton & Miller (1950) to separate the epimeric 5α:6α- and 5β:6β-epoxycholestan-3β-ols. Pilot experiments with synthetic mixtures of 20-epimeric benzoates showed at once that these could be separated almost quantitatively in a single chromatogram on alumina, the 20β-benzoate being eluted before the 20α. (These pilot experiments have been described briefly by Brooks, Klyne & Miller, 1951*b*; a fuller account of this work is in preparation. Turner & Voitle (1951) described the use of benzoates for separating 20-epimeric steroids almost simultaneously with us.)

All those portions of fraction β1(*E*) which had not been separated as pure uranediol or 5α-pregnane-3β:20β-diol were benzoylated and chromatographed. The chromatograms yielded four major constituents

as dibenzoates in the following order: 5α-pregnane-3β:20β-diol, 5α-pregnane-3β:20α-diol, uranediol and a new *compound MM*. All these benzoates have sharp melting points and crystallize well; a solvent suitable for crystallizing many steroid benzoates is chloroform-methanol (20:80 v/v). This appears to be the first occasion in which a pair of 20-epimeric hydroxysteroids has been isolated from the same urine. Data proving the identity of the 5α-pregnane-3β:20α-diol from mares' urine with authentic material are given in Table 4. The constitution of compound *MM* has not been elucidated. From the analysis of its benzoate and from the chromatographic behaviour of the alcohol and the benzoate it seems probable that it is an isomer of pregnanediol ($C_{21}H_{36}O_2$) or pregnenediol ($C_{21}H_{34}O_2$). It is not identical with pregnane-3β:20β-diol or 5α-pregn-16-ene-3β:20β-diol, which it resembles in some properties (see Table 5).

Fraction β2(E). This was chromatographed on alumina after the removal of material insoluble in benzene. Most of the material was eluted with benzene-ether mixtures; the earlier fractions yielded almost pure uranediol, the later fractions, crude 5α-pregnane-3β:20β-diol. As was the case with fraction β1(*E*) the compounds could be separated much more easily as benzoates than as alcohols. Thus uranediol and 5α-pregnane-3β:20β-diol appeared in both β1 and β2 fractions; the 5α-pregnanediol chiefly in the β1 fraction and the uranediol in the β2 fraction.

The benzene-insoluble portion of fraction β2(*E*) was chromatographed after benzoylation. 5α-Pregnane-3β:20β-diol dibenzoate was eluted first, followed by a fraction consisting of the tribenzoates of the triols—chiefly 5α-pregnane-3β:16α:20β-triol.

Quantities isolated. It is impossible after such a lengthy fractionation process to estimate the quantities of the various steroids originally present in the urine, but the quantities of the principal steroid diols isolated in a fair state of purity from 270 l. urine are as follows. (The figures include material isolated as alcohols, as benzoates and as sulphate, all calculated as alcohols.) Uranediol, 390 mg.; 5α-pregnane-3β:20β-diol, 250 mg.; 5α-pregnane-3β:20α-diol, 20 mg.; compound *MM*, 40 mg.

Fraction α(E). This has not been investigated fully, but the benzene-insoluble portion of it yielded a new polymorphic modification of uranetriol triacetate.

The triol fraction

This fraction contained no phenols and very little ketonic material. The non-ketonic portion has yielded two compounds, namely 5α-pregnane-3β:16α:20β-triol (first isolated by Haslewood, Marrian & Smith, 1934; constitution determined by Hirschmann, Hirschmann & Daus, 1949) and uranetriol (Marker, Kamm, Crooks, Oakwood, Wittle & Lawson, 1938; Marker, Kamm, Oakwood, Wittle & Lawson, 1938). No generally satisfactory method of separating these triols has yet been found. The two isomers could be separated from one batch of extract by fractional crystallization of their acetates from methanol and aqueous methanol (Marker, Kamm, Crooks *et al.* 1938). The identity of our uranetriol triacetate (m.p. 135–136°) and that of Marker (m.p. 136°) was proved by the identity of their infrared absorption spectra which were kindly determined by Dr K. Dobriner and Mrs P. Humphries of the Sloan-Kettering Institute, New York. The triol fraction from the other batch of extract could not be separated by chromatography and fractional crystallization of the acetates in this way. However, fractional crystallization of the mixed acetates from light petroleum followed by methanol yielded a compound of m.p. 153–154°; this was shown by the identity of its infrared absorption in carbon disulphide with that of uranetriol triacetate (m.p. 135–136°) to be a polymorphic modification of the latter. Both forms of uranetriol triacetate on alkaline hydrolysis gave uranetriol, the melting point of which agreed with that found by Marker, Kamm, Crooks *et al.* (1938).

After it had been found that steroid diols, which could not be separated efficiently by chromatography of their acetates, could often be separated well by chromatography of their benzoates, attempts were made to separate the two triols in this way. These attempts were unsuccessful except where one or other triol predominated in the mixture; in such cases the predominant isomer could be obtained pure.

EXPERIMENTAL

Material

The material used consisted of all the 'water-insoluble' conjugate fractions (Klyne *et al.* 1948), which had not been separated as sulphates of 5α-pregn-16-en-3β-ol-20-one, 5α-pregnane-3β-ol-20-one and uranediol, and the 'water-soluble' conjugate fractions from two batches of pooled late pregnancy urine. Batch *B* (270 l. urine) was obtained from the Ovaltine Research Laboratories and British Drug Houses Ltd.; batch *D* (530 l. urine) was obtained from N.V. Organon, Oss, Netherlands.

General methods

Melting points. These were determined on the hot-stage apparatus of Klyne & Rankeillor (1947), and are corrected for emergent stem.

Specific rotations. These were determined for the *D* line, using a 0·5 dm. microtube. The errors are calculated as described by Klyne *et al.* (1948) except that five pairs of readings with the solutions and five pairs with a solvent blank were taken in each case. Temperature, where not stated, was 20–25°.

Micro-analyses. These are by Dr J. W. Minnis, Edinburgh, or Drs Weiler and Strauss, Oxford. Compounds were dried to constant weight at 110° *in vacuo* unless stated otherwise.

Ultraviolet absorption spectra. These were determined in ethanol (sometimes containing 1% (v/v) $CHCl_3$), using a Beckman spectrophotometer, model DU.

'*Usual working-up.*' See Klyne (1948).

Chromatograms. These were carried out using Al_2O_3 as described by Paterson & Klyne (1948). The proportions of mixed solvents are given as % (v/v). The activity of the Al_2O_3 was determined according to Brockmann & Schodder (1941), and was II–III where not stated otherwise.

Light petroleum was the fraction of b.p. 60–80°.

Benzoates. These were prepared by dissolving the steroid in pyridine (1 ml./100 mg.), cooling the solution in ice and adding benzoyl chloride dropwise (0·1 ml./100 mg. of steroid for each hydroxyl group). The solution was allowed to stand for 18 hr. at room temperature and then ice was added to react with the excess benzoyl chloride. After being allowed to stand for a further 2–3 hr. the mixture was worked up in the usual way.

Acetates. These were prepared in the same way as the benzoates using acetic anhydride (0·2 ml./100 mg. of steroid) in place of benzoyl chloride.

Hydrolysis of conjugates

The following notes describe the hydrolysis and fractionation of batch *D*. Batch *B* was treated in a similar way, except for the digitonin separation described on p. 701. The 'water-insoluble' and 'water-soluble' fractions were combined in 2·5 l. water; conc. HCl (200 ml., 11 N) was added and the mixture was heated at 100° for 2 hr. It was cooled and extracted with ether (2 × 500 ml., 1 × 300 ml., 3 × 200 ml.); a little tarry material which adhered to the sides of the separating funnels was dissolved in ethanol and added to the ether extracts. The aqueous solution was rejected and the ethereal extracts used for the separation of phenols from non-phenols.

Separation of the major steroid fractions

Phenolic and non-phenolic fractions. This separation is shown diagrammatically in Fig. 2.

Since weak phenols cannot be readily extracted with N-NaOH from ether, but can be extracted by this reagent from benzene, the 'non-phenolic' fraction I from the ether was transferred to benzene and then was further extracted. This method was very clumsy, due to the unexpected appearance of a benzene-insoluble fraction (later called the triol fraction). In future we propose to extract the original hydrolysis mixtures with benzene, filter off the benzene-insoluble material, and extract the benzene solutions with N-NaOH.

The products finally obtained were: triol fraction, 0·7 g. pale-buff solid; non-phenolic fraction II, 5·54 g. dark-brown gum; phenolic fractions (I and II), 1·50 g. pinkish sticky crystalline solid. The triol fraction gave a negative Millon reaction, and therefore contained little or no phenolic material.

Non-phenolic ketones and non-ketones. The non-phenolic fraction was separated further using the hydrazide reagent T of Girard & Sandulesco (1936) in the conditions described by those authors. The first Girard separation yielded a ketonic fraction (2·25 g.) and a non-ketonic fraction (2·82 g.). Each of these fractions was submitted to a further Girard treatment since this process does not effect a clean-cut separation. The final ketonic fraction weighed 1·59 g. and the final non-ketonic fraction 2·39 g.

Separation of non-phenolic non-ketones by digitonin in methanol

The final non-ketonic fraction from the Girard separation of batch *D* was submitted to digitonin separation in 90% methanol as described by Shoppee (1946) yielding the following fractions: non-ketonic, precipitated by digitonin; fraction β (*M*), 968 mg.; non-ketonic, not precipitated by digitonin, fraction α(*M*), 843 mg.

Fraction β(M)

This fraction (968 mg.) was dissolved in benzene and chromatographed on 30 g. Al_2O_3 (activity I–II) giving the following fractions (solvents used for elution are given in parentheses). *A* (benzene), 30 mg.; *B* (benzene-ether mixtures), 727 mg.: *C* (ether), 15 mg.; *D* (acetone), 42 mg.; *E* (methanol), 39 mg.

The main fraction *B* from the first chromatogram was acetylated and the acetates dissolved in 5 ml. benzene and 95 ml. hexane and chromatographed on Al_2O_3 (27 g., activity IV). The following fractions were obtained: *BA* (hexane:benzene, 95:5), 253 mg. gum; *BB* (hexane:benzene, 95:5), 340 mg. crystalline solid; *BC* (hexane:benzene, 50:50 and 40:60), 169 mg. crystalline solid; *BD* (benzene), 33 mg. partly crystalline; *BE* (ether), 62 mg. gum.

Compound MV. Fraction *A* was again chromatographed on 1 g. Al_2O_3 (activity IV). Pentane eluted 27 mg. partly crystalline material, which when crystallized twice from methanol-ether formed leaflets, m.p. 61–63°. This substance, provisionally called *compound MV*, was readily soluble in ether or $CHCl_3$ and sparingly soluble in methanol. It gave a precipitate with digitonin in 90% (v/v) ethanol. It gave no colour with tetranitromethane in $CHCl_3$, or in the Liebermann-Burchard test. It was sublimed for analysis at 90–115°/3 μ. (Found, C, 84·1; H, 13·7. $C_{21}H_{36}O$ requires C, 82·9; H, 11·9. $C_{27}H_{48}O$ requires C, 83·5; H, 12·4. $C_{30}H_{50}O$ requires C, 84·6; H, 11·7. $C_{30}H_{52}O$ requires C, 84·2; H, 12·0%.) Lack of material prevented further work on this compound.

Compound MU diacetate. Fraction *BB* was crystallized from hexane (4 ml.) giving 66 mg. of crystals (fraction *BB*1), m.p. 150–165°. This material after three further crystallizations from hexane yielded 7·5 mg. of thick plates, m.p. 202–206° after subliming at about 180° and softening at 197°. This material, provisionally called *compound MU diacetate* was sublimed at 160–180/1 μ. (Found C, 74·4; H, 9·1. $C_{35}H_{38}O_4$ requires C, 74·6; H, 9·5. $C_{35}H_{40}O_4$ requires C, 74·2; H, 10·0%.) $[\alpha]_D^{22°} - 89·5 \pm 1·0°$ in $CHCl_3$ (*c*, 0·58). If molecular formula is $C_{35}H_{38}O_4$, $[M]_D$ is −360.

The ultraviolet absorption of compound *MU* diacetate in the region 200–220 mμ. was measured with a Unicam SP500 spectrophotometer (at Birkbeck College, University of London, through the courtesy of Dr D. H. R. Barton). The compound showed the following values for ϵ (assuming the molecular formula $C_{35}H_{38}O_4$): at $\lambda_{max.}$, 203 mμ.; ϵ, 2240: at 210 mμ.; ϵ, 810: at 220 mμ.; ϵ, 87. These values are compatible with the presence of an olefinic linkage, probably trisubstituted (cf. Bladon *et al.* 1951; Halsall, 1951). The compound gave a faint but definite yellow colour with tetranitromethane in $CHCl_3$.

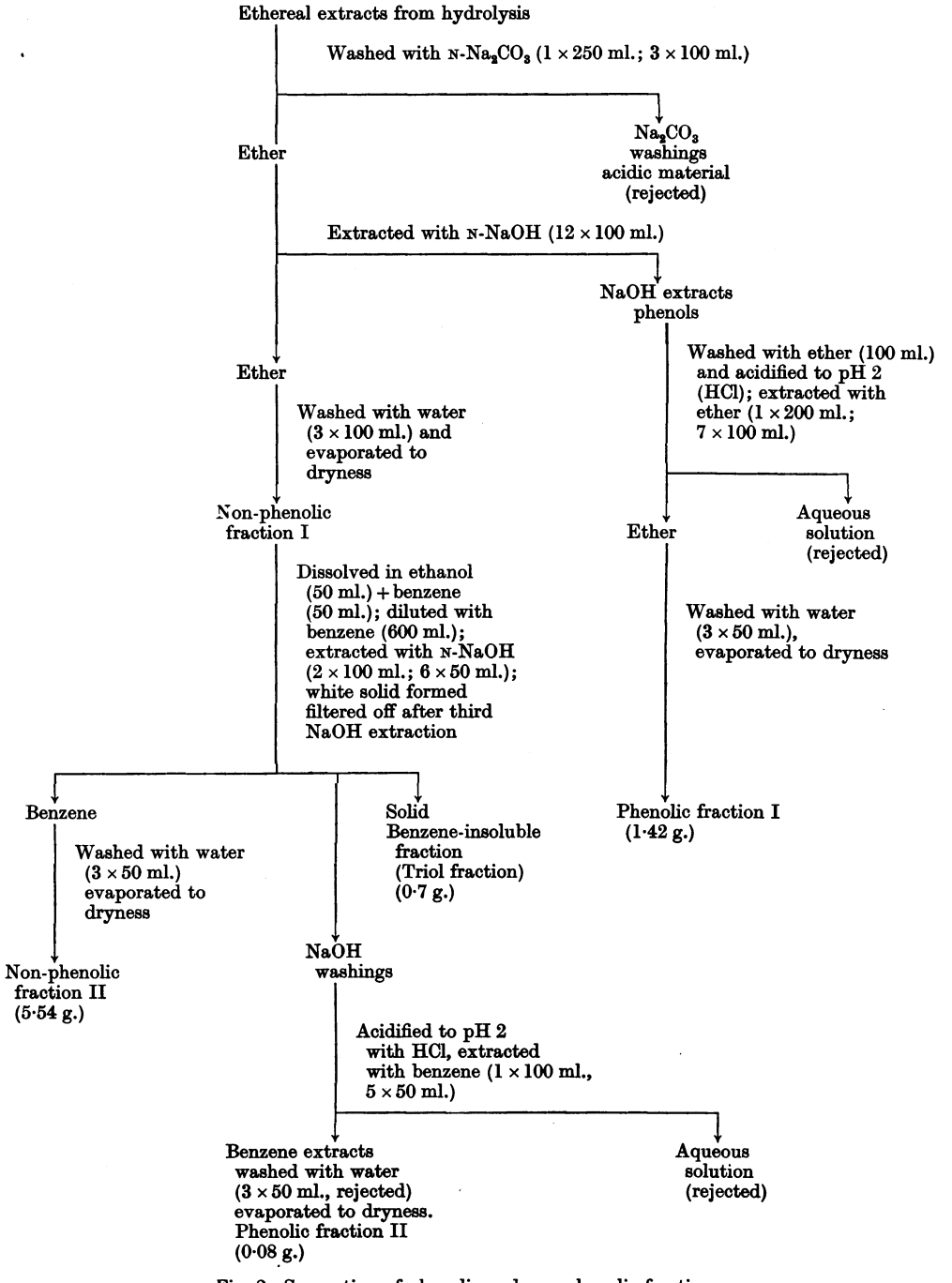


Fig. 2. Separation of phenolic and non-phenolic fractions.

Uranediol diacetate. The mother liquors of fraction *BB*1 after further chromatography and crystallization from hexane and from aqueous methanol yielded some uranediol diacetate. Fraction *BA* and all the material from fraction *BB* which had not been purified as uranediol diacetate or compound *MU* were combined and hydrolysed with KOH; the alcohols were submitted to the Marker digitonin separation (as described on p. 701). Uranediol was obtained pure in this way very much more easily than by chromatography and crystallization of the acetates.

Compound MW diacetate. Fraction *BC* (169 mg.) was recrystallized twice from aqueous methanol, once from aqueous ethanol and twice from hexane to give a product, rectangular leaflets, m.p. 175·5–178°, provisionally called *compound MW diacetate.* (Found after subliming at 150–180°/1 μ., C, 74·8; H, 9·7. $C_{25}H_{38}O_4$ requires C, 74·6; H, 9·5. $C_{25}H_{40}O_4$ requires C, 74·2; H, 10·0%. $[\alpha]_D^{19°}$ −54·0±1·1° in $CHCl_3$ (*c*, 1·03).) Insufficient pure material was available for a duplicate determination of the rotation, but since the first determination gave an unexpectedly large negative value, a second determination was carried out, using less pure material of m.p. 173–176°. The value found, $[\alpha]_D^{19°}$ −46·0 ±0·6° in $CHCl_3$ (*c*, 1·00), was in rough agreement with the previous value. *MW* diacetate gave a barely perceptible colour with tetranitromethane in $CHCl_3$.

chromatographed on Al_2O_3. The material eluted with light petroleum-benzene mixtures (70:30–50:50), after crystallization from $CHCl_3$-methanol, formed leaflets, m.p. 214–218° (softening from 209°). This product gave a considerable depression in melting point on admixture with uranediol dibenzoate of m.p. 211–213°. The product has $[\alpha]_D^{20°}$ −24·9 ±0·5° in $CHCl_3$ (*c*, 0·82). The ultraviolet absorption showed maxima at 228 and 272 mμ.; $E_{1\,cm.}^{1\%}$ 317 and 20·2. These values are far too low for the dibenzoate of a C_{21} diol; Pearlman (1944) found $E_{1\,cm.}^{1\%}$ 525 and 37·6 for the corresponding maxima for 5α-pregnane-3β:20β-diol dibenzoate. Cholestan-3β-ol benzoate (mol.wt. 492) had $\lambda_{max.}$ 228 and 272 mμ., $E_{1\,cm.}^{1\%}$ 284 and 17·1 (unpublished observations by Mr H. S. Wiggins). The $E_{1\,cm.}^{1\%}$ values calculated from this for a C_{21} acetate-benzoate of mol.wt. 466 are 300 and 18·1, with

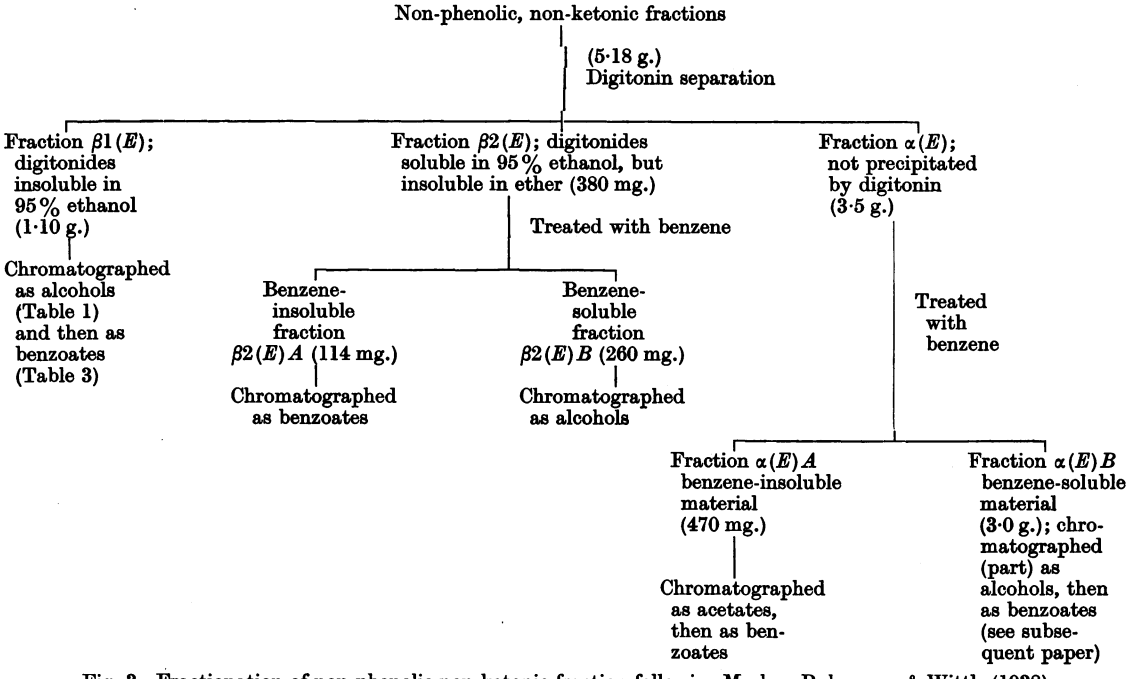


Fig. 3. Fractionation of non-phenolic non-ketonic fraction following Marker, Rohrmann & Wittle (1938). Outline of method.

Hydrolysis of compound MW diacetate with potassium carbonate. The diacetate (8 mg.) in methanol (0·5 ml.) was hydrolysed by refluxing with K_2CO_3 (10 mg.) in water (0·1 ml.) for 1 hr. The usual working-up yielded a product which was readily soluble in ethanol, sparingly soluble in hexane, more soluble in ether; this on crystallization from hexane-ether gave rosettes of very small needles, m.p. 152–156°. This material gave no precipitate with digitonin in 90% ethanol, and no colour with tetranitromethane in $CHCl_3$. It dissolved in conc. H_2SO_4 to give, after a few seconds, an orange-red colour with a slight green fluorescence; in the Liebermann-Burchard reaction both layers developed a golden-yellow colour in a few seconds. Evidence presented later indicates that this material may have been compound *MW* monoacetate.

The mother liquors of the crystallization from which *MW* diacetate had been obtained were hydrolysed with K_2CO_3 in aqueous methanol; the crude product was benzoylated and which the experimental values for the benzoylation product of m.p. 214–218° are in fair agreement.

Hydrolysis of MW esters with potassium hydroxide. The presumed acetate-benzoate of m.p. 214–218° (18 mg.) was refluxed for 2 hr. with excess KOH in aqueous methanol. The usual working-up yielded 10 mg. solid which, after crystallization from ether-light petroleum, gave small needles m.p. 215° (after subliming in needles from 190°). Mixed m.p. with uranediol, 212–215°. The product gave, during heating on the Kofler block, the characteristic smell of heated uranediol. Concentration of the mother liquors gave a further quantity of the same material. $[\alpha]_D^{20°}$ +2·9 ±1·0° in $CHCl_3$ (*c*, 0·62). Two previous determinations of the rotation of uranediol gave $[\alpha]_D$ +2·1±0·8° and +3·7±0·8° in $CHCl_3$.

The hydrolysis product on acetylation gave an acetate which on crystallization from methanol formed prisms, m.p. 163–165°; not depressed on admixture with authentic uranediol diacetate.

The presumed monoacetate of compound *MW* (m.p. 152–156°) was similarly hydrolysed with KOH in aqueous methanol. The product had m.p. 214–217°, not depressed by admixture with uranediol.

Separation of non-phenolic non-ketones by digitonin in ethanol and ether

The non-ketonic material from batch *B* (5·18 g.) was dissolved in boiling ethanol (95% v/v, 450 ml.) and a hot solution of digitonin (15 g.) in ethanol (95% v/v, 900 ml.) was added (cf. Marker, Rohrmann & Wittle, 1938). The separations are outlined in Fig. 3. The mixture was cooled to 15–20° and after 3 hr. the precipitate which formed was filtered off, washed with ethanol (95% v/v) and decomposed with pyridine and ether. The digitonin-free product (fraction $\beta 1(E)$) weighed 1·10 g. The ethanolic filtrate was concentrated *in vacuo* on the water bath until solid began to separate (volume of solution, approx. 40 ml.). Dry ether (800 ml.) was added and the precipitate which formed was filtered off after standing 1 hr., and washed with ether. This second digitonide precipitate was decomposed like the first, giving fraction $\beta 2(E)$ (380 mg.). The ethereal filtrate was evaporated to dryness giving fraction $\alpha(E)$ (3·5 g.).

Chromatography of fraction $\beta 1(E)$ as free alcohols

The fraction (1·10 g.) was dissolved as much as possible in benzene-ether (80:20), filtered from a little undissolved material, and chromatographed on Al_2O_3. The results of this and four subsequent chromatograms on the material as free alcohols are shown in Table 1.

Uranediol. Fraction III*B* (Table 1) after crystallization from aqueous ethanol or from ethyl acetate-light petroleum gave uranediol, 50 mg., m.p. 216–219°, not depressed by admixture with an authentic sample obtained from the sulphate (Klyne, 1948). Further quantities (30 mg.) were obtained from fractions II*C* and *D* by chromatography and crystallization.

5α-Pregnane-3β:20β-diol. Each of the seven groups of fractions IV*B–H* (Table 1) on crystallization from ethyl acetate-light petroleum yielded a product of melting point between 186 and 194°, total wt. 170 mg. The three best fractions were combined and crystallized again from the

Table 1. *Chromatography of fraction $\beta 1(E)$ as alcohols*

Run	Eluent	Eluate: Fraction no.	Eluate: Weight (mg.)	Eluate: Appearance	Melting point
	Chromatogram I: Fraction $\beta 1(E)$; 30 g. Al_2O_3; each run 100 ml. of eluent				
1–3	Benzene-ether (80:20)	I*A*	352	Gum, see chromatogram II	—
4	Benzene-ether (80:20)	I*B*	219	Solid, see chromatogram III	182–192°
5–9	Benzene-ether (80:20)	I*C*	376	Solid, see chromatogram IV	155–175°
10–12	Benzene-ether (50:50)	I*D*	53	Gum } see chromatogram V	—
13–14	Ether	I*E*	26	Gum }	—
15–17	Ether-methanol (50:50) and methanol	I*F*	240	Gum	—
	Chromatogram II: Fraction I*A*; 10·5 g. Al_2O_3; each run 35 ml. of eluent				
1–5	Benzene-ether (90:10)	II*A*	133	Gum	—
6–7	Benzene-ether (90:10)	II*B*	25	Crystals	170–190°
8–9	Benzene-ether (80:20)	II*C*	32	Crystals	203–210°
10	Benzene-ether (80:20)	II*D*	8	Crystals	193–205°
11–25	Benzene-ether (60:40 and 20:80) and ether	II*E*	12	Gum	—
	Chromatogram III: Fraction I*B*; 18 g. Al_2O_3; each run 20 ml. of eluent				
1–11	Benzene-ether (up to 25% ether)	III*A*	21	Gum	—
12–13	Benzene-ether (70:30)	III*B*	80	Crystals	207–217°
14	Benzene-ether (70:30)	III*C*	29	Crystals	190–200°
15	Benzene-ether (70:30)	III*D*	33	Crystals	145–160°
16–18	Benzene-ether (70:30)	III*E*	39	Crystals	155–180°
19–20	Benzene-ether (60:40)	III*F*	20	Gum	—
	Chromatogram IV: Fractions I*C* + III*E*; 27 g. Al_2O_3; each run 40 ml. of eluent				
1–8	Benzene-ether (85:15 and 80:20)	—	Nil		—
9–11	Benzene-ether (75:25)	IV*A*	113	Crystals	150–180°
12–15	Benzene-ether (75:25)	IV*B–H*	309	Seven groups of crystalline fractions	165–190° mainly 180–190°
16–23	Benzene-ether (70:30)				
24–25	Benzene-ether (60:40)	IV*J*	19	Crystals	150–165°
	Chromatogram V: Fractions I*D* + I*E* + II*E*; 2·2 g. Al_2O_3; each run 7 ml. of eluent				
1–2	Benzene-ether (66:33)	V*A*	23	Gum	—
3–5	Benzene-ether (66:33)	V*B*	13	Gum	—
6–9	Benzene-ether (33:66)	V*C*	5	Solid	90–110°
10–12	Ether	V*D*	12	Gum	—
13–26	Ethanol-methanol mixtures and methanol				

Table 2. *Identification of urinary 5α-pregnane-3β:20β-diol*

	Urinary compound		Authentic 5α-pregnane-3β:20β-diol*
Diol			
M.p.	194·5–196°		194–195°
Mixed m.p.		195–198°	
$[\alpha]_D$ in $CHCl_3$	−0·7±0·7° (*c*, 1·11)		+2·9±0·9° (*c*, 1·02)
Analysis	C, 78·7; H, 11·2%		C, 78·7; H, 11·3% (calc. for $C_{21}H_{36}O_2$)
Diacetate			
M.p.	140·5–141·5°		141–142°
Mixed m.p.		139·5–141°	
$[\alpha]_D$ in $CHCl_3$	+20·8±0·8° (*c*, 1·00)		+22° (*c*, 5·2)
Analysis	C, 74·0; H, 10·1%		C, 74·2; H, 10·0% (calc. for $C_{25}H_{40}O_4$)
Dibenzoate			
M.p.	236·5–237·5°		237·5–239°
Mixed m.p.		235·5–237·5°	
$[\alpha]_D$ in $CHCl_3$	−7·7±0·4° (*c*, 2·04)		−10·1±0·5° (*c*, 2·08)
Analysis	C, 79·6; H, 8·6%		C, 79·5; H, 8·4% (calc. for $C_{35}H_{44}O_4$)

* Data for the diol are from Klyne & Miller (1950); data for the diacetate from Klyne & Barton (1949); data for the *dibenzoate* are new.

Table 3. *Chromatography of parts of fraction β1 (E) as benzoates*

Run	Eluent	Fraction no.	Eluate: Weight (mg.)	Eluate: Appearance*	Melting point
Chromatogram VI: Benzoylated mother liquors of 5α-pregnane-3β:20β-diol from fractions IV *B–H* (Table 1). 12 g. Al_2O_3; each run 20 ml. of eluent					
1–8	Light petroleum-benzene (90:10)	VI *A*	82	Crystals (5α-pregnane-3β:20β-diol)	230–240°
9–13	Light petroleum-benzene (85:15)	VI *B*	32	Solid (5α-pregnane-3β:20α-diol)	158–170°
14–16	Light petroleum-benzene (80:20)				
17–19	Light petroleum-benzene (70:30)	VI *C*	4	Trace gum	—
20–25	Light petroleum-benzene (50:50)	VI *D*	35	Solid (compound *MM*)	145–157°
26–29	Light petroleum-benzene (25:75)	VI *E*	23	Gum	—
30–32	Benzene				
Chromatogram VII: Fractions II *D*, III *C*, *D*, *F*, IV *A*, Table 1 (intermediate between uranediol and 5α-pregnane-3β:20β-diol in alcohol chromatograms); benzoylated; 15 g. Al_2O_3; each run 25 ml. of eluent					
1–3	Light petroleum-benzene (95:5)	VII *A*	10	Gum	—
4–13	Light petroleum-benzene (90:10)	VII *B*	111	Solid (5α-pregnane-3β:20β-diol)	234–238°
14–16	Light petroleum-benzene (85:15)	VII *C*	13	Solid (as VII *B*)	223–232°
17	Light petroleum-benzene (85:15)	VII *D*	18	Solid	120–216°
18–20	Light petroleum-benzene (80:20)				
21	Light petroleum-benzene (70:30)				
22–25	Light petroleum-benzene (70:30)	VII *E*	54	Solid (uranediol)	202–211°
26	Light petroleum-benzene (70:30)	VII *F*	16	Solid	135–197°
27	Light petroleum-benzene (60:40)				
28–32	Light petroleum-benzene (60:40)	VII *G*	29	Solid (compound *MM*)	183–196°
33–40	Light petroleum-benzene (50:50 and 25:75) and benzene	VII *H*	22	Gum	—

* Compounds present, as benzoates, shown in parentheses.

same solvent mixture yielding 40 mg. 5α-pregnane-3β:20β-diol. The properties of this product and its diacetate, and of authentic 5α-pregnane-3β:20β-diol are listed in Table 2.

Chromatography of fraction β1 (E) as acetates

Fraction I*F* (Table 1) was the material eluted last from Al_2O_3: it was expected that this would consist of triols. It was acetylated and chromatographed on Al_2O_3. Light petroleum-benzene mixtures (60:40 and 50:50) eluted solid fractions (56 mg.) with melting points in the range of 126–155°. This material was combined and recrystallized twice from methanol (0·5 and 0·3 ml.) and once from light petroleum (0·5 ml.) to yield 5α-pregnane-3β:16α:20β-triol triacetate, m.p. 164–168° (not depressed by admixture with authentic material), $[\alpha]_D^{20°} - 51·3 \pm 0·6°$ in $CHCl_3$ (*c*, 1·35).

m.p. 163–164°, which depressed the m.p. of 5α-pregnane-3β:20α-diol dibenzoate. A second recrystallization from the same mixture (about 4 ml.) gave rectangular leaflets (15 mg.) which were a polymorphic modification of the compound, m.p. 191·5–192·5°. (Found: C, 79·7; H, 8·1. $C_{35}H_{44}O_4$ requires C, 79·5; H, 8·4. $C_{35}H_{42}O_4$ requires C, 79·8; H, 8·0. $C_{33}H_{40}O_4$ requires C, 79·2; H, 8·1. $C_{33}H_{38}O_4$ requires C, 79·5; H, 7·7%.) The ultraviolet absorption of *MM* benzoate was compatible with its formulation as the dibenzoate of a C_{21} diol; $\lambda\lambda_{max.}$ 230 and 273 mμ., $E_{1\,cm.}^{1\%}$, 527 and 39·0. Pearlman (1944) found for 5α-pregnane-3β:20β-diol dibenzoate $\lambda\lambda_{max.}$ 231 and 272 mμ., $E_{1\,cm.}^{1\%}$ 525 and 37·6.

Recrystallization of fraction VII*G* from $CHCl_3$-methanol mixture yielded a further 18 mg. of compound *MM* benzoate.

Table 4. *Identification of urinary 5α-pregnane-3β:20α-diol*

	Urinary compound		Authentic 5α-pregnane-3β:20α-diol*
Dibenzoate			
M.p.	170·5–172°		170–171°
Mixed m.p.		168·5–169·5°	
$[\alpha]_D^{20°}$ in $CHCl_3$	+27·7±0·7° (*c*, 0·84)		+27·8±0·2° (*c*, 2·10)
Diol			
M.p.	217–218°		218–220°
Mixed m.p.		217–218·5°	
$[\alpha]_D$ in $CHCl_3$	+20·0±1·4° (*c*, 0·64)		+22·6±1·3° (*c*, 0·93)

* 5α-*Pregnane*-3β:20α-*diol dibenzoate* (authentic). (Found: C, 79·2; H, 8·3. $C_{35}H_{44}O_4$ requires C, 79·5; H, 8·4%.)

Chromatography of fraction β1 (E) as benzoates

The mother liquors (116 mg.) from the crystallization of 5α-pregnane-3β:20β-diol in fractions IV *B–H* (Table 1) were benzoylated and the product (190 mg.) chromatographed on Al_2O_3. (Chromatogram VI, Table 3.)

The mother liquors from the crystallization of uranediol were combined with fractions II*D*, III*C*, *D*, *F*, and IV*A* (Table 1) and benzoylated. The product (260 mg.) was chromatographed on Al_2O_3. (Chromatogram VII, Table 3.)

From these chromatograms four benzoates were readily separated, namely those of 5α-pregnane-3β:20α- and -3β:20β-diols, uranediol and a new compound *MM*.

5α-*Pregnane*-3β:20β-*diol*. Fraction VI*A* on recrystallization twice from $CHCl_3$-methanol (25:75) yielded needles, m.p. 236·5–237·5°. Details of the comparison with authentic 5α-pregnane-3β:20β-diol dibenzoate and analysis are given in Table 2. Further quantities were obtained from fractions VII*B*, *C* and VIII *B*, *C* by crystallization.

5α-*Pregnane*-3β:20α-*diol*. Fraction VI*B* was recrystallized twice from $CHCl_3$-methanol (20:80) yielding 12 mg. of prisms which proved to be the dibenzoate of 5α-pregnane-3β:20α-diol. The properties of this product, of its parent diol and of authentic material are listed on Table 4. Dr K. Dobriner (Sloan-Kettering Institute, New York) kindly determined the infrared absorption of the dibenzoate obtained from the urinary material and stated that it was identical with that of the authentic material 'with the exception of a small band at 1040 cm.$^{-1}$, which may be due to some impurity'.

Compound MM. Recrystallization of fraction VI*D* (Table 3) from $CHCl_3$-methanol mixture (20:80, about 6 ml.) yielded 24 mg. of *compound MM dibenzoate*, plates,

Hydrolysis of the benzoate (24 mg.) by refluxing with methanolic KOH yielded 13·5 mg. of a product which was recrystallized twice from ethyl acetate-light petroleum to yield compound *MM* (5·3 mg.), rosettes of elongated prisms, m.p. 184–185° after subliming in needles from 162°. Compound *MM* gave a precipitate with digitonin in 95% ethanol, but gave no colour with tetranitromethane. In the Liebermann-Burchard test it rapidly gave a reddish brown colour in the conc. H_2SO_4 layer; the $CHCl_3$ layer remained colourless.

Compound *MM* was acetylated and the product was chromatographed on Al_2O_3 and recrystallized from light petroleum giving needles, m.p. 108–109·5°.

Table 5 shows a comparison of the properties of compound *MM* and its derivatives with those of pregnane-3β:20β-diol and 5α-pregn-16-ene-3β:20β-diol. *MM* is clearly not identical with pregnane-3β:20β-diol. Dr K. Dobriner kindly examined the infrared absorption of *MM* acetate; he stated that it was not identical with that of 5α-pregn-16-ene-3β:20β-diol diacetate, but that compound *MM* may contain an olefinic double bond.

Uranediol. Fraction VII*E* (Table 3) was recrystallized twice from $CHCl_3$-methanol (20:80) to yield leaflets (30 mg.), m.p. 209–210°, not depressed by admixture with an authentic sample of uranediol dibenzoate, $[\alpha]_D^{29°} + 18·6° \pm 0·6°$ (in $CHCl_3$, *c*, 1·05). The authentic sample of the *dibenzoate* had m.p. 211–213°. $[\alpha]_D + 22·4° \pm 1·2°$. (Found: C, 79·4; H, 8·1. $C_{35}H_{44}O_4$ requires C, 79·5; H, 8·4%.) Ultraviolet absorption, $\lambda_{max.}$ 230 and 272 mμ., log $\epsilon_{max.}$ 4·43 and 3·26.

Other compounds. Fraction II*A* (Table 1; 133 mg.) consisted of material more easily eluted from Al_2O_3 than the pregnanediol isomers. It was chromatographed again on Al_2O_3 but, since no apparent separation occurred, the bulk

of the material was combined and benzoylated. Chromatography of this product on Al_2O_3 yielded chiefly gums, but small quantities of two solid compounds were obtained. The first solid fraction (4 mg.) eluted with light petroleum-benzene (90:10) was recrystallized twice from methanol to yield plates of m.p. 167–171° (compound *MR* benzoate). The second compound was eluted with light petroleum-benzene (60:40) mixtures and had m.p. 185–188° after being recrystallized twice from methanol (compound *MS* benzoate). Neither compound was identical with any known benzoate.

eluted *BR* (50 mg.) and methanol *BS* (50 mg.). Since uranediol and 5α-pregnane-3β:20β-diol were subsequently obtained more easily by chromatography of their benzoates, it seems unnecessary to give details of their isolation as diols from these fractions.

The mother liquors from the crystallization of 5α-pregnane-3β:20β-diol and uranediol were combined with similar material from the urine of batch *D* and benzoylated. The product (179 mg.) was chromatographed on Al_2O_3. Light petroleum-benzene mixtures (85:15) eluted 70 mg. of solid which was recrystallized from $CHCl_3$-methanol to yield 5α-

Table 5. *Comparison of compound MM with pregnane-3β:20β-diol and 5α-pregn-16-ene-3β:20β-diol*

	Compound *MM*		Pregnane-3β:20β-diol	5α-Pregn-16-ene-3β:20β-diol†
Dibenzoate				
M.p.	163–164° and 191·5–192·5°		Gum	—
$[\alpha]_D$ in $CHCl_3$	−14·6±0·4° (*c*, 1·04)		−13° (calc.)*	—
Analysis	C, 79·7; H, 8·1%		C, 79·5; H, 8·4% (calc. for $C_{35}H_{44}O_4$)	C, 79·8; H, 8·0% (calc. for $C_{35}H_{42}O_4$)
Diol				
M.p.	184–185°		177–178°‡	188–190°
Mixed m.p.		169–178°		
$[\alpha]_D$ in EtOH	−5·9±0·7° (*c*, 0·55)		+4·9±0·4° (*c*, 1·91)	—
Diacetate				
M.p.	108–109·5°		108–110°‡	102–104°
Mixed m.p.		104–110°		
$[\alpha]_D$ in $CHCl_3$	−14·0±0·7° (*c*, 0·59)		+32·9±0·8° (*c*, 1·04)	—

* From tables of standard values given by Barton & Klyne (1948).

† Data from Marker, Turner, Wagner, Ulshafer, Crooks & Wittle (1941).

‡ Authentic pregnane-3β:20β-diol was prepared from pregnan-3β-ol-20-one by reduction with $LiAlH_4$ as described for pregn-5-en-3β-ol-20-one by Klyne & Miller (1950) except that the reaction mixture was not heated. The 3β:20α- and 3β:20β-diol dibenzoates were separated by chromatography. Marker, Kamm & Jones (1937) and Marker, Kamm, Wittle *et al.* (1937) give for pregnane-3β:20β-diol m.p. 174°, and for its diacetate m.p. 111°.

The bulk of the material from chromatogram V (fractions V*A*, *B* and *C*) was benzoylated and chromatographed on Al_2O_3 (chromatogram VIII). Nearly all the material was eluted with light petroleum-benzene mixtures. The first four 10% benzene runs eluted a little 5α-pregnane-3β:20β-diol dibenzoate (fractions VIII*B* and *C*). Runs 6 and 7 (10% benzene) eluted 11 mg. crystalline material which after two recrystallizations from $CHCl_3$-methanol formed plates m.p. 185·5–187°. This material was apparently different from all the other benzoates isolated in this work and has been called provisionally compound *MN* benzoate. It gave a depression in melting point on admixture with the benzoate of similar melting point (*MS*) from fraction II*A* above. Lack of material has prevented further study. Later runs with increasing benzene content (10–50%) eluted material melting chiefly below 100°, from which no homogeneous product has been obtained.

Fraction β2(E)

This fraction (380 mg.) was treated with benzene (50 ml.) and separated into a benzene-insoluble fraction β2(*E*) *A* (114 mg.) and a benzene-soluble fraction β2(*E*) *B* (266 mg.).

Benzene-soluble fraction. Fraction β2(*E*) *B* was chromatographed on Al_2O_3: benzene-ether mixtures (75:25) eluted first fraction *BP* (46 mg., fairly pure uranediol of m.p. 205–207°) and then *BQ* (70 mg.); this was a mixture containing uranediol and 5α-pregnane-3β:20β-diol; acetone

pregnane-3β:20β-diol dibenzoate. Further solid fractions were eluted with 20% benzene (10 mg.) and 30% benzene (34 mg.); these on recrystallization yielded the dibenzoates of 5α-pregnane-3β:20α-diol and uranediol, respectively.

Fraction *BR* was benzoylated and the product chromatographed on Al_2O_3. The first solid fraction eluted (3 mg.) with light petroleum-benzene (80:20) was recrystallized from $CHCl_3$-methanol to yield plates, m.p. 181–184°. (Mixed melting point with compound *MN* benzoate—from chromatogram VIII—183–187°.) Fraction *BS* was benzoylated and the product chromatographed on Al_2O_3. Light petroleum-benzene mixtures (80:20 and 70:30) eluted solid fractions (79 mg.) with indefinite melting points in the range 100–140°. Crystallization of these fractions has not, so far, yielded a homogeneous product.

Benzene-insoluble fraction. Fraction β2(*E*)*A* was benzoylated and the product (183 mg.) chromatographed on Al_2O_3 (chromatogram IX). The principal solid fractions, eluted with light petroleum-benzene mixtures, were as follows. Fraction IX*B*, eluted with 20% benzene (8 mg.) contained 5α-pregnane-3β:20β-diol dibenzoate. Fraction IX*D* (25 and 30% benzene; 32 mg.) and fraction IX*F* (40% benzene, 54 mg.) yielded the tribenzoate of 5α-pregnane-3β:16α:20β-triol after one crystallization from methanol as elongated prisms m.p. 211–214°, not depressed by admixture with an authentic sample. The intermediate fraction IX*E* (30 and 40% benzene, 48 mg.) consisted of less pure 5α-pregnane-3β:16α:20β-triol tribenzoate.

Fraction α(E)

This was separated, by warming with 30 ml. benzene and filtering, into two fractions, *α(E)B* (soluble in benzene, 3·0 g.) and *α(E)A* (insoluble in benzene, 470 mg.). Fraction *α(E)B* has been kept for further study; fraction *α(E)A* was treated like the triol fraction from batch *D* (see below).

The triol fraction

Separation of acetates by crystallization from methanol. The triol fraction from batch *D* (0·7 g.) (see p. 698) was acetylated and the product submitted to the Girard separation. The ketonic fraction (28 mg.) has not been further investigated. The non-ketonic fraction (767 mg.) was chromatographed on Al_2O_3 (activity II). Hexane-benzene mixtures (60:40 and 40:60) eluted white, crystalline fractions, which were all apparently mixtures. These fractions (*TA*) were combined (440 mg.) and recrystallized twice from methanol (2 ml. and 1 ml.) (cf. Marker, Kamm, Crooks *et al.* 1938) giving 177 mg. of needles, m.p. 158–160°. After a further recrystallization from methanol this material had m.p. 164–166°. The mixed m.p. with a sample of 5α-pregnane-3β:16α:20β-triol triacetate of Haslewood *et al.* (1934), m.p. 163–165°, kindly supplied by Prof. Marrian, was 163–164°. The methanol mother liquors from the first crystallization of the fractions *TA* (1·5 ml.) were treated with water (0·5 ml.), warmed to dissolve the precipitate which formed and then cooled. The crystalline solid which separated (200 mg., m.p. 134–137°) was recrystallized from 70% methanol-30% water giving uranetriol triacetate, small plates, m.p. 135–136°. Marker, Kamm, Crooks *et al.* (1938) give m.p. 136°. This substance sublimed at 180°/0·5 μ. (Found: C, 69·6; H, 9·2. Calc. for $C_{27}H_{42}O_6$: C, 70·1; H, 9·2%.) $[\alpha]_D^{21°}$ +7·8°±1·3° in $CHCl_3$ (*c*, 1·00); $[\alpha]_D^{21°}$ +0·0°±0·9° in ethanol (*c*, 1·01). $[M]_D$ in $CHCl_3$, +37.

Separation of uranetriol triacetate by crystallization from light petroleum. It was expected that fraction *α(E)A* from batch *B* (see above) would consist of triols; this fraction (470 mg.) was therefore acetylated and the acetates (560 mg.) chromatographed on Al_2O_3. Light petroleum-benzene mixtures and benzene eluted 430 mg. gum (fraction *TB*); benzene-ether and ether eluted approx. 130 mg. yellow gum (fraction *TC*). Fraction *TB* was chromatographed again yielding 370 mg. almost colourless, partly crystalline material (some fractions melted at approx. 80–90°). This material failed to crystallize from methanol when the Marker fractionation of triacetates was attempted. However, when the material was allowed to stand at 0° with 1 ml. light petroleum (b.p. 40–60°) for a week, crystals formed. The supernatant liquid was removed and the crystals triturated twice with 0·5 ml. light petroleum. The final crystalline product (103 mg.; m.p. 123–125°) was crystallized three times from methanol and once from ethyl acetate-light petroleum yielding a compound m.p. 153–154° (15 mg.). (Found, on material sublimed at 170–180°/0·1 μ., C, 69·7; H, 8·8. Calc. for $C_{27}H_{42}O_6$: C, 70·1; H, 9·1%) $[\alpha]_D^{22°}$ +12·2°±0·6° in $CHCl_3$ (*c*, 1·00). Subsequent comparison of the infrared absorption of this material (form *b*) and of our previous uranetriol triacetate (form *a*; m.p. 135–136°) by Dr K. Dobriner and Mrs P. Humphries showed that the two substances were identical. Mixed melting point determinations with uranetriol triacetate (form *a*) were inconclusive. Form *a* melted at about 128–131° and remained liquid on further heating; form *b* melted at 151–153°.

Separation of uranetriol tribenzoate by chromatography. Mother liquors from the crystallization of uranetriol triacetate from urine of batch *B* were hydrolysed, benzoylated and then chromatographed as their benzoates (293 mg.). The first two fractions eluted with light petroleum-benzene mixture (60:40) were gums (53 mg.). These were followed by four solid fractions (108 mg.), m.p. 199–230°, eluted with the same mixture. Repeated recrystallization of these solid fractions from $CHCl_3$-methanol gave *uranetriol tribenzoate* as fine needles, m.p. 233·5–235°, undepressed by a specimen prepared from an authentic sample of uranetriol. $[\alpha]_D^{20°}$ + 82·5°±0·4° in $CHCl_3$. Another sample had $[\alpha]_D$ +80·8±0·4° in $CHCl_3$ (*c*, 1·02) $[M]_D$ +524. (Found, C, 77·3; H, 7·5. $C_{42}H_{46}O_6$ requires C, 77·7; H, 7·5%.) Ultraviolet absorption, $\lambda_{max.}$ 231 and 272 mμ., log $\epsilon_{max.}$ 4·61 and 3·48.

Uranetriol. Samples of the two forms of the triacetate (*a*, of m.p. 135–136°; *b*, of m.p. 153–154°) were hydrolysed with boiling aqueous methanolic KOH. The products obtained by the usual working-up and crystallized from acetone-methanol were solids of no definite crystalline appearance. Triol from the triacetate (form *a*) had m.p. 296–298° (uncorr.) after subliming to long needles at 230° on the Kofler block. $[\alpha]_D^{20°}$ +29·8±3·1° in ethanol (*c*, 0·41). The triol from the triacetate (form *b*) had m.p. 297–299° (uncorr.) after subliming at 235°, $[\alpha]_D^{22°}$ +28·5±1·5° in ethanol (*c*, 0·53); another sample $[\alpha]_D^{20°}$ +26·2±1·4° (*c*, 0·56); $[M]_D$ +94. Mixed melting point of triols from the two forms of the triacetate, 294–297° (uncorr.). Marker, Kamm, Crooks *et al.* (1938) give m.p. 295–300° for uranetriol. Uranetriol gave no precipitate with digitonin in 90% (v/v) ethanol and no coloration with tetranitromethane. In conc. H_2SO_4 it gave an orange solution with a yellow-green fluorescence and in the Liebermann-Burchard test an orange colour.

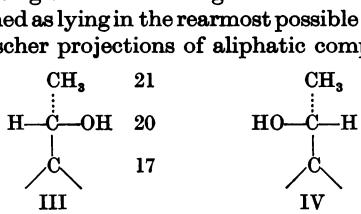
5α-Pregnane-3β:16α:20β-triol and derivatives. 5α-Pregnane-3β:16α:20β-triol obtained from the triacetate by hydrolysis with methanolic KOH had m.p. 309–310° (uncorr.), $[\alpha]_D^{22°}$ −16·2±1·7° in ethanol (*c*, 0·47), $[M]_D$ −54. Haslewood *et al.* (1934) give $[\alpha]_{5461}^{20°}$ −44·1° in pyridine. The triacetate had $[\alpha]_D^{23°}$ −45·3±0·6° in $CHCl_3$ (*c*, 1·07), $[\alpha]_D^{25°}$ −45·7°±0·6° in ethanol (*c*, 1·01); $[M]_D$ −208.

The tribenzoate formed long thin needles from $CHCl_3$-methanol, m.p. 211–213° (Marker, Kamm, Wittle, Oakwood & Lawson, 1938, give m.p. 218°) $[\alpha]_D^{20°}$ −14·1±0·8° in $CHCl_3$ (*c*, 1·06), $[M]_D$ −91. (Found: C, 77·3; H, 7·5%. Calc. for $C_{42}H_{46}O_6$: C, 77·7; H, 7·5%.)

DISCUSSION

Configurations at $C_{(20)}$

Configurations at $C_{(20)}$ in the steroid skeleton in this paper are allotted following Fieser & Fieser (1948, 1949) who first related the stereochemistry of $C_{(20)}$ to that of the nucleus. They proposed a convention for representing side-chain configurations in which $C_{(21)}$ is imagined as lying in the rearmost possible position, as in Fischer projections of aliphatic compounds:



They produced evidence which indicated that, for compounds carrying a 17α-hydroxyl group, the 20α- and 20β-configurations were as in III and IV. This allotment of configurations was extended to compounds not carrying a 17α-hydroxyl group by Sarett (1949) and by Klyne & Barton (1949). The arguments of Fieser & Fieser regarding these configurations were recently called in question by Lardon & Reichstein (1951); evidence confirming the Fieser configurations was subsequently discussed by Klyne (1951).

The occurrence of 20β-hydroxysteroids in urine

The chief result of this work to date is the confirmation of the finding by Bauld & Heard (1940) that 5α-pregnane-3β:20β-diol is one of the major steroids of mares' pregnancy urine. No 20β-hydroxysteroid has ever been reported in human pregnancy urine, in spite of the fact that the 'pregnanediol' fractions have been studied by many workers. (For review, see Pearlman, 1948.) Mares' pregnancy urine contains another 20β-hydroxysteroid—the 5α-pregnane-3β:16α:20β-triol isolated by Haslewood *et al.* (1934), the structure of which was finally elucidated by Hirschmann *et al.* (1949). Two triols of somewhat similar structure have recently been identified in the urine of a boy with an adrenocortical tumour (Hirschmann & Hirschmann, 1950*a*, *b*). These compounds, pregn-5-ene-3β:16α:20α-triol and -3β:17α:20α-triol, like all other human urinary steroids carrying a hydroxyl group at $C_{(20)}$, have the 20α-configuration.

Marker & Rohrmann (1938) claimed to have isolated 5α-pregnane-3β:20α-diol from pregnant mares' urine, but they obtained this compound only from a fraction which had been epimerized by heating with sodium in xylene. We found a much smaller quantity of 5α-pregnane-3β:20α-diol than of the 20β-isomer, and it is surprising that Marker & Rohrmann did not isolate the 20β-isomer.

At an early stage in this work we suspected that Marker's 3β:20α-diol might be an artifact formed from the 3β:20β-diol by epimerization at $C_{(20)}$. However, our own experiments in which 5α-pregnane-3β:20β-diol was heated with sodium in xylene confirmed the findings of Marker, Kamm, Wittle, Oakwood, Lawson & Laucius (1937), who stated that no appreciable epimerization at $C_{(20)}$ takes place in these conditions.

The present work appears to be the first occasion on which a pair of 20-epimeric hydroxysteroids has been isolated from the same urine. The only other source from which a pair of 20-epimers has been obtained is the adrenal glands, from which Reichstein (1936) and Steiger & Reichstein (1938) isolated 5α-pregnane-3β:17α:20α-triol and -3β:17α:20β-triol ('substances *O* and *J*').

Two 20β-hydroxysteroids have been isolated from ox bile, namely 5α-pregnane-3β:20β-diol (Pearlman, 1944, 1946) and pregnane-3α:20β-diol (from pregnant cows' bile, Pearlman & Cerceo, 1948).

Value of alternative digitonin separations

The Marker digitonin separation using ethanol and ether (Marker, Rohrmann & Wittle, 1938) offers a considerable advantage over the digitonin separation using methanol in that the two principal 3β-hydroxysteroids of the mares' urine extracts (5α-pregnane-3β:20β-diol and uranediol) are partly separated, one being concentrated in each of the two digitonin precipitates of the Marker method. The separation is, however, far from perfect, and before the benzoates were used in chromatography, the separation of the two major constituents in pure condition was difficult, and the separation of the minor constituents almost impossible.

The fact that some minor constituents (compounds *MU*, *MV* and *MW*) have been isolated only from the urine extracts submitted to the methanol-separation, whilst others (compounds *MM*, *MN*, *MR* and *MS*) have been isolated only from the extracts treated by the Marker method may be due to differences in the solubilities of the acetates and benzoates of the compounds, or to chance differences in the composition of the two batches of pooled urine. It is possible that *MU* or *MW* (obtained as their acetates) may be identical with *MN*, *MR* or *MS* (obtained as their benzoates), but the quantities of material available at present do not enable us to test these hypotheses.

Although neither of the two triols (5α-pregnane-3β:16α:20β-triol and uranetriol) is precipitated from pure solution by digitonin in the usual conditions, the fractionation of batch *B* by the Marker digitonin method shows that the 5α-pregnanetriol may be partially precipitated from mixtures by digitonin.

SUMMARY

1. The fractionation of the steroids of pregnant mares' urine into phenols and non-phenols, ketones and non-ketones, and the further separation of some fractions with digitonin are described.

2. The non-phenolic, non-ketonic, digitonin-precipitable fraction has been studied in detail. The isolation of uranediol, 5α-pregnane-3β:20β-diol and 5α-pregnane-3β:20α-diol from this fraction is described. This is the first reported isolation of a pair of 20-epimeric hydroxysteroids from any urine.

3. The isolation of seven apparently new compounds of undetermined structure from the same fraction is described. Compounds *MM*, *MU* and *MW* are probably C_{21} dihydroxysteroids; they were

isolated as their acetates or benzoates. Compound *MW* may be a 17*a*-epimer of uranediol. Compound *MV* is apparently a monohydroxy compound. The three remaining compounds, *MN*, *MR* and *MS*, have been obtained only in traces.

4. The significance of the isolation of 20β-hydroxysteroids from mares' urine is discussed. All 20-hydroxysteroids obtained from human urine are 20α-compounds.

5. Uranetriol has been obtained from the trihydroxysteroid fraction and studied.

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