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The Structure of Urinary Oestriol Monoglucuronide

BY J. G. D. CARPENTER* AND A. E. KELLIE
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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A conjugate of oestriol [oestra-1,3,5(10)-triene-3,16α,17β-triol] was first isolated from human-pregnancy urine by Cohen & Marrian (1936). The compound is probably a monoglucuronide in which the phenolic hydroxyl group at C-3 in the steroid is free (Cohen, Marrian & Odell, 1936; Grant & Marrian, 1948, 1950). It has not been established, however, whether glucuronic acid is linked to oestriol at the C-16 or C-17 hydroxyl group.

The present work concerns the synthesis of derivatives of the two possible ring D monoglucuronides of oestriol, and the comparison of these compounds with corresponding derivatives of oestriol glucuronide isolated from late-pregnancy urine. A preliminary account has been presented (Carpenter & Kellie, 1961).

EXPERIMENTAL

Materials

Solvents, except ethanol, were distilled in an all-glass apparatus before use. Celite 535 (Johns Manville and Co. Ltd.) was covered with hot conc. HCl for 24 hr., filtered, washed thoroughly with water until free from chloride, and dried at 120°. The neutral alumina (Merck) was sieved to exclude particles < 200 mesh. β-Glucuronidase was prepared as 'powder B', of activity 10^6 units/g., from the visceral hump of the limpet (*Patella vulgata*) according to Dodgson & Spencer (1953). Diazomethane was obtained from *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide (Diazald; Aldrich Chemical Co. Inc.) by the method of de Boer & Backer (1954). Methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucuronate (bromo ester) was prepared according to Pelzer (1959), and stored under vacuum over P_2O_5 in the dark at 5°.

Methods

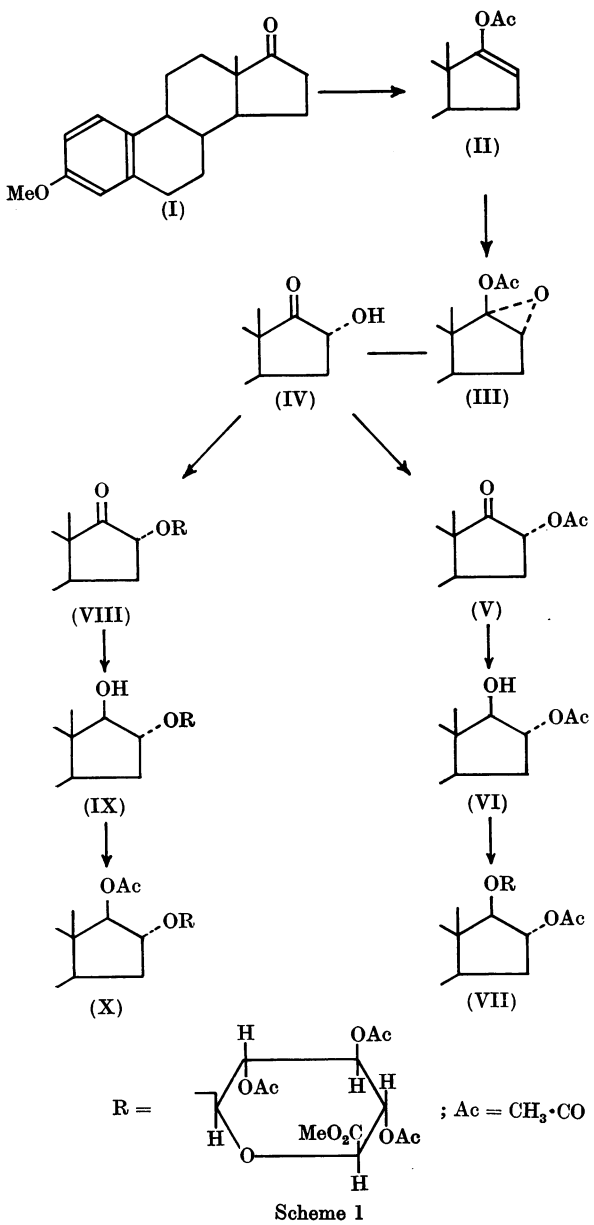
Paper chromatograms were run on unwashed Whatman no. 2 paper by the descending method (Bush, 1952). Oestriol and its derivatives were detected by a modification of the Kober reaction (Ittrich, 1958). Melting points were determined on the Kofler hot-stage and are corrected. Infrared-absorption spectra were measured on a Perkin–Elmer Model 21 recording spectrophotometer with sodium chloride optics. Optical rotations were determined in ethanol. Elementary analyses were carried out by Weiler and Strauss, Oxford.

Synthesis of the two isomeric derivatives of oestriol glucuronide

Methyl [16α-acetoxy-3-methoxyoestra-1,3,5(10)-trien-17β-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate (VII) and methyl [17β-acetoxy-3-methoxyoestra-1,3,5(10)-trien-16α-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate (X) were synthesized from oestrone methyl ether [3-methoxyoestra-1,3,5(10)-trien-17-one] (I) by the route outlined in Scheme 1. The enol acetate (II), the epoxide (III) and the α-ketol (IV) were synthesized by methods similar to those described in the preparation of the analogous C-3 acetates by Leeds, Fukushima & Gallagher (1954). The enol acetate (II) has been described by Johnson & Johns (1957); the preparation of the α-ketol (IV) has been reported by Mueller & Johns (1961).

3-Methoxyoestra-1,3,5(10)-trien-17-one (I). Dimethyl sulphate (15 ml.) was added during 15 min. to a stirred boiling solution of oestrone (2·54 g.) in methanol (45 ml.) and aq. 10% (w/v) NaOH (90 ml.). The mixture was refluxed for 1·5 hr., and then treated with further quantities of 10% (w/v) NaOH (30 ml.) and dimethyl sulphate (15 ml.) during 15 min. The suspension was refluxed for 1 hr., cooled, diluted to 300 ml. with water and filtered. The yield of oestrone methyl ether (m.p. 169·5–170°) was 2·6 g. (98%).

* Present address: Department of Obstetrics and Gynaecology, Columbia University, College of Physicians and Surgeons, New York, 32 N.Y., U.S.A.



Scheme 1

17-*Acetoxy*-3-*methoxyoestra*-1,3,5(10),16-*tetraene* (II). The methyl ether (I) (7·0 g.) was treated with isopropenyl acetate, in the presence of catalytic amounts of sulphuric acid, as described in the preparation of 3,17-diacetoxyoestra-1,3,5(10),16-tetraene (Leeds *et al.* 1954). The yield of 17-*acetoxy*-3-*methoxyoestra*-1,3,5(10),16-*tetraene* (m.p. 108–110°) was 6·44 g. (80%). A sample crystallized twice from methanol had m.p. 114–114·5°. Johnson & Johns (1957) report m.p. 114·5–115°.

17β-*Acetoxy*-16α,17α-*epoxy*-3-*methoxyoestra*-1,3,5(10)-*triene* (III). The enol acetate (II) (6·0 g.) was treated with excess of perbenzoic acid in dry benzene (cf. Leeds *et al.* 1954). The yield of 17β-*acetoxy*-16α,17α-*epoxy*-3-*methoxyoestra*-1,3,5(10)-*triene* was 3·73 g. (59%). A sample crystallized three times from methanol had m.p. 147·5–149° and $[\alpha]_D^{21}$ +72·6±0·5° (*c* 0·523) (Found: C, 73·4; H, 7·75. $C_{21}H_{26}O_4$ requires C, 73·7; H, 7·65%).

16α-*Hydroxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17-*one* (IV). A solution of (III) (3·22 g.) in a mixture of methanol (600 ml.) and acetone (100 ml.) was treated with 6N-H_2SO_4 (120 ml.) and left at room temperature for 6 days. After dilution with an equal volume of chilled (3°) ethyl acetate, the solution was washed with cold saturated $NaHCO_3$ soln. containing crushed ice, and then with ice-cold water. The organic phase was dried over Na_2SO_4 and the solvent removed under reduced pressure at 40°. The residue was crystallized from ethanol to yield 2·0 g. (71%) of 16α-*hydroxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17-*one*, m.p. 148–150°. The analytical sample (crystallized successively from aqueous ethanol and propanol) had m.p. 152–153° and $[\alpha]_D^{21}$ +147±1° (*c* 0·675) (Found: C, 75·8; H, 8·1. Calc. for $C_{19}H_{24}O_3$: C, 76·0; H, 8·05%). Mueller & Johns (1961) record m.p. 156–157° and $[\alpha]_D$ +176° (*c* approx. 1 in chloroform).

16α-*Acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17-*one* (V). The α-ketol (IV) (2·05 g.) was treated with acetic anhydride (100 ml.) and pyridine (50 ml.) at room temperature overnight. The reaction mixture was poured into chilled 3N-H_2SO_4 (650 ml.). The precipitate was filtered off and washed with water until free from acid. The yield of 16α-*acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17-*one* (m.p. 155–156°) was 2·32 g. (99%). After three crystallizations from methanol, the analytical specimen had m.p. 157·5–158° and $[\alpha]_D^{21}$ +164±0·5° (*c* 0·418) (Found: C, 73·9; H, 7·5. $C_{21}H_{26}O_4$ requires C, 73·7; H, 7·65%).

16α-*Acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17β-*ol* (VI). A solution of (V) (1·8 g.) in methanol (260 ml.) was cooled to 5° and treated with a cold solution of sodium tetrahydroborate (borohydride) (0·91 g.) in methanol (30 ml.). The mixture was kept at 5° for 10 min. Acetic acid was added until no more gas was evolved and the mixture was poured into water (600 ml.) containing acetic acid (10 ml.). The flocculent white precipitate was filtered off, washed copiously with water and dried. 16α-*Acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17β-*ol* separated from aqueous ethanol as a mass of minute needles (1·36 g.; 75% yield), m.p. 168–171° (slight decomp.). Crystallization from aqueous ethanol, in a vessel contained in a beaker of hot (75°) water, yielded larger needles. The analytical specimen had m.p. 170–171·5°, $[\alpha]_D^{21}$ +12·9±0·8° (*c* 0·398) and $\gamma_{max.}^{CS_2}$ 1722, 3560 cm.$^{-1}$ (Found: C, 72·9; H, 7·9. $C_{21}H_{28}O_4$ requires C, 73·2; H, 8·2%).

A sample of (VI) was acetylated with acetic anhydride and pyridine in the usual way. The infrared spectrum of the product in carbon disulphide was identical with that of an authentic specimen of 16α,17β-diacetoxy-3-methoxyoestra-1,3,5(10)-triene.

Methyl [16α-*acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17β-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* (VII). Dry freshly-prepared Ag_2CO_3 (1·17 g.) was suspended in a boiling solution of (VI) (500 mg.) in anhydrous benzene (20 ml.). A solution of bromo ester (1·52 g.) in dry benzene (20 ml.) was added dropwise during 20 min.; meanwhile solvent was distilled off at the same rate. The mixture was cooled and the violet–black silver salts were removed by

filtration. The salts were washed well with chloroform, and the combined yellow filtrate and washings evaporated to dryness under reduced pressure. The residual oil was triturated with cold ether and kept at 2° overnight. The fine needles of *methyl* [16α-*acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-17β-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* (58 mg.; 6% yield) which separated after two recrystallizations from ethanol had m.p. 169–170° and $[\alpha]_D^{21}$ −21·2± 1° (*c* 0·417) (Found: C, 61·8; H, 6·5. $C_{34}H_{44}O_{13}$ requires C, 61·8; H, 6·7%).

Methyl [3-*methoxy*-17-*oxo*-*oestra*-1,3,5(10)-*trien*-16α-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* (VIII). The α-ketol (IV) (2·0 g.) was converted into (VIII) by the method described for the preparation of (VII) with Ag_2CO_3 (5·0 g.) and bromoester (7·0 g.). *Methyl* [3-*methoxy*-17-*oxo*-*oestra*-1,3,5(10)-*trien*-16α-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* (1·5 g.; 36·5% yield) separated from ethanol as clumps of fine needles, m.p. 216–217°, raised to 217–218° after two crystallizations from ethanol, and $[\alpha]_D^{21}$ +71·8±1° (*c* 0·105) (Found: C, 62·6; H, 6·45. $C_{32}H_{40}O_{12}$ requires C, 62·3; H, 6·5%).

Methyl [17β-*hydroxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-16α-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* (IX). The triacetylglucuronic acid methyl ester (VIII) (143 mg.) was reduced with sodium tetrahydroborate in methanol at 5° as described in the preparation of (IV). *Methyl* [17β-*hydroxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-16α-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* (114 mg.; 79% yield) separated from ethanol as a mass of fine white needles, m.p. 208–211°. The analytical specimen (crystallized three times from ethanol) had m.p. 214–216·5°, $[\alpha]_D^{21}$ +12·7±0·8° (*c* 0·141) and $\gamma_{max.}^{CS_2}$ 3590 cm.$^{-1}$ (Found: C, 62·2; H, 6·5. $C_{32}H_{42}O_{12}$ requires C, 62·1; H, 6·8%).

Methyl [17β-*acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-16α-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* (X). A solution of (IX) (140 mg.) in pyridine (5 ml.) was treated with acetic anhydride (10 ml.) in the usual way. *Methyl* [17β-*acetoxy*-3-*methoxyoestra*-1,3,5(10)-*trien*-16α-*yl* 2,3,4-*tri*-O-*acetyl*-β-D-*glucopyranosid*]*uronate* crystallized from ethanol as thick needles (120 mg.; 80·5% yield), m.p. 165–166° and 180–182°. After two crystallizations from ethanol, the analytical sample had m.p. 165·5–167° and 180–182°, and $[\alpha]_D^{21}$ −22·4±1° (*c* 0·235) (Found: C, 61·6; H, 6·9. $C_{34}H_{44}O_{13}$ requires C, 61·8; H, 6·7%).

Isolation of oestriol glucuronide from human pregnancy urine

Late-pregnancy urine (36th week; 2600 ml., containing 37 mg. of oestriol) was extracted with butan-1-ol as described by Cohen (1950). The final butanol extract was concentrated to about 300 ml. under reduced pressure. The extract was resolved into free steroid, sulphate and glucuronide fractions by chromatography on alumina (Crépy, Jayle & Meslin, 1957*a*, *b*). Thus the concentrate was run on to a column (2·9 cm. × 10 cm.) of alumina (100 g.) packed in dry butanol. The column was washed with butanol containing increasing amounts of water; the glucuronide fraction was finally eluted with 0·1 N-NH_3. The ammoniacal butanol was evaporated to dryness and the residue applied to dry Celite 535 (2 g.) in methanol. The Celite was dried in the desiccator, mixed with lower phase (1·2 ml.) from the solvent system butan-1-ol–toluene–acetic acid–water (5:15:6:14, by vol.), and packed on a column (2 cm. × 38·8 cm.) of Celite 535 (60 g.) prepared in the same system. Fractions (each 10 ml.) were numbered to include the first retention volume (56·9 ml.).

The eluate was examined both by paper chromatography, with the monophasic system described by Lewbart & Schneider (1955), and by the Kober reaction. Oestriol glucuronide was found in fractions 70–115 (maximum, 88). The contents of these tubes were combined and evaporated to dryness, giving a white non-crystalline solid.

Methylation and acetylation of the glucuronide

A preliminary study of the action of an ethereal solution of diazomethane on oestrone indicated that the phenolic hydroxyl group in the steroid was only partly methylated under the conditions used to esterify carboxylic acids. Dimethyl sulphate, on the other hand, readily methylates phenols under mild conditions (cf. Brown, 1955). Diazomethane and dimethyl sulphate were therefore used in conjunction to ensure effective methylation.

The oestriol glucuronide was dissolved in methanol (5 ml.) to which was then added 3 N-NaOH (5 ml.). The solution was stirred mechanically and cooled to about 5°. Dimethyl sulphate (2 ml.) was added in small portions during 30 min. The cooling bath was removed and the mixture stirred at room temperature overnight. The strongly acidic solution was diluted with water to 100 ml. and adjusted to pH 2. Ammonium sulphate (50 g.) was dissolved in the solution, which was then extracted with 3 × 50 ml. of ether–ethanol (3:1, v/v). The extract was evaporated to dryness, the residue dissolved in methanol and treated with excess of a freshly prepared solution of diazomethane in ether at room temperature overnight. The solvents were removed on the rotary evaporator and the residue was treated with acetic anhydride (3 ml.) and pyridine (2 ml.) overnight at room temperature. The excess of reactants was distilled off under reduced pressure, with frequent additions of methanol. The derivative was then applied to dry Celite 535 (2 g.) in methanol, and the solvent removed in the vacuum desiccator.

The Celite bearing the derivative was mixed with lower phase (1·2 ml.) from the 'solvent system A' of Bush (1952) and packed on a column (2 cm. × 37·1 cm.) of Celite 535 (60 g.) prepared in the same system. Examination of the fractions (each 10 ml.) by the modified Kober reaction revealed two peaks of material. The first peak was contained in fractions 10–18 (maximum, 15) and the second in fractions 21–30 (maximum, 25). The second was eluted with the same volume of mobile phase as that in which either of the two synthetic derivatives (VII) and (X) was eluted from a similar column. A portion of fraction 25 was examined by paper chromatography in the 'solvent system A' of Bush (1952). Only one spot was revealed when the paper was treated with phosphomolybdic acid at 95° (Lewbart & Schneider, 1955). The contents of the tubes containing the second peak were combined and evaporated to dryness. The residual film of gum was dissolved in ethanol and transferred to a small conical centrifuge tube; the solvent was removed under a stream of nitrogen at 60°. Trituration of the residual gum with cold light petroleum (b.p. 60–80°) gave a fleecy white solid almost immediately. Most of the solvent was removed with a Pasteur pipette and the final traces were evaporated under a stream of nitrogen. The residue (4·6 mg.; m.p. 146–155°) was dissolved in the minimum volume of boiling propan-2-ol and set aside for 15 min. The solution set to a gel containing

isolated centres of small needles. The mixture was diluted with about 1 vol. of ether, stirred and chilled in ice–water. Tiny needles separated smoothly during the next 40 min. The mixture was centrifuged, and the supernatant liquid was removed with a Pasteur pipette and added to the light petroleum used in the trituration. The dry solid in the centrifuge tube was crystallized from ethanol, from which it separated as long needles (1·0 mg.) (compound A). The combined mother liquors and trituration solvent were evaporated to yield a white solid which separated from ethanol as blunt needles (3·1 mg.) (compound B).

RESULTS AND DISCUSSION

The two synthetic derivatives (VII) and (X) of oestriol glucuronide had very similar properties. The infrared-absorption spectra of the two compounds, both in carbon disulphide solution and in potassium bromide disks, were essentially identical. Each spectrum contained a strong C=O stretching vibration at 1756 cm.$^{-1}$, due to the large proportion of ester groups in the molecule. Equally strong C—O and C—C stretching vibrations in the same groups dominated the characteristic region at 1214 and 1238 cm.$^{-1}$, masking nearly all of the absorption bands associated with the steroid part. It is all but impossible to distinguish between steroid triacetylglucuronic acid methyl esters unless the steroid contains functional groups which absorb in the infrared in the carbonyl region at frequencies well removed from 1756 cm.$^{-1}$ (e.g. C-11 ketones), or at the low-frequency end of the characteristic region (e.g. benzyloxy compounds).

The specific rotations of the two isomers (VII) and (X) were identical within the limits of experimental error. The two isomers could barely be resolved by paper chromatography. In the 'solvent system A' of Bush (1952) at 35° the compounds (VII) and (X) had R_F values of 0·56 and 0·50 respectively. When run in the same track on the chromatogram, the compounds ran as a single elongated spot. The derivatives were also chromatographed in the low-temperature system methanol–light petroleum (b.p. 100–120°) (1:1, v/v) described by Edwards (1960); they could not be completely resolved after development for 64 hr. at 2°.

Compounds (VII) and (X) differed, however, in crystalline form and in melting behaviour. One isomer (VII) separated from ethanol as fine matted needles, which were only slightly birefringent in polarized light, and melted at 169–170°; the other isomer (X) was obtained as short blunt needles, which were markedly anisotropic in polarized light. This compound had two distinct melting points; the original crystals melted at 165·5–167° and from the liquid grew a network of long blades, which then melted at 180–182°.

There was a good correspondence in crystalline form, behaviour in polarized light, and in melting point between compound A (169–170°) and compound (VII), and between compound B (166–166·5° and 181–183°) and compound (X). The mixed melting points were: A+(VII), 168–169°; B+(VII), 163–170°; B+(X), 165–166° and 180–181°; A+(X), 164·5–170°. The specific rotations of compound A [$-22·4 \pm 3°$ (*c* 0·143)] and compound B [$-21·7 \pm 3°$ (*c* 0·230)] were identical, within the limits of experimental error, and were in good agreement with the value found for each of the synthetic isomers.

Although the derivatives of urinary oestriol glucuronide and the corresponding synthetic isomers have been compared by only one reliable criterion, melting behaviour, this evidence, together with the similarity of crystalline form and degree of birefringence in polarized light, indicates that both ring D isomers of oestriol glucuronide occur in late-pregnancy urine. The amounts of crystalline triacetylglucuronic acid methyl esters obtained suggest that there was three times as much oestriol 16α-glucuronide as oestriol 17β-glucuronide in the sample of pregnancy urine examined. The 17β-conjugate may be less abundant than the 16α-glucuronide as the result of steric hindrance of the 17-β-hydroxyl group by the angular methyl group at C-13 during glucuronide formation.

The nature of the Kober-positive substance eluted in the first peak from the Celite partition column has not been investigated, but it is thought to be an artifact, as similar secondary products have often been obtained in this Laboratory after methylation and acetylation of other steroid glucuronides.

Felger & Katzman (1961) found that an oestriol diglucuronide also occurs in human-pregnancy urine, although the relative proportions or the precise nature of the mono- and di-conjugates have not yet been recorded. The apparently poor yield of oestriol glucuronide derivatives obtained in the present work may be due to the presence of significant amounts of the much more polar diglucuronide, which would have been discarded during the chromatographic purification of the urinary extract.

Neeman & Hashimoto (1961) investigated the structure of urinary oestriol monoglucuronide by an elegant degradative procedure and found only the 16α-glucuronide. The conjugate was crystallized three times before methylation and acetylation, however, and the 17β-glucuronide may have been lost during the initial purification.

SUMMARY

1. Derivatives of the two possible ring D isomers of oestriol glucuronide, methyl [16α-acetoxy-3-methoxyoestra-1,3,5(10)-trien-17β-yl

2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate and methyl [17β-acetoxy-3-methoxyoestra-1,3,5(10)-trien-16α-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate, have been synthesized from oestrone.

2. Oestriol glucuronide was extracted from human-pregnancy urine with butanol, and purified by chromatography on an alumina column and a Celite partition column.

3. This glucuronide was methylated and acetylated and resolved into two components by fractional crystallization.

4. Comparison of the two components with the two synthetic isomers indicated that both ring D glucuronides of oestriol monoglucuronide exist in human-pregnancy urine.

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The Isolation of Mouse Antigens Carrying H2-Histocompatibility Specificity: Some Preliminary Studies

BY D. A. L. DAVIES
Microbiological Research Establishment, Porton, Salisbury, Wilts.

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The rejection of tissue transplants, whether grafts of normal or malignant tissue, is the result of an immunological reaction on the part of the recipient against antigens present in the donor cells (Medawar, 1958*a*). Although transplantation studies are now being carried out with a variety of mammalian and avian species as inbred lines become available, the genetic system underlying the distribution of transplantation (T) antigens is best understood in the mouse (Snell, 1957).

In mice, antigens governing histocompatibility are determined by several genetic loci, H1, H2, H3 etc. Because H2 antigens are generally 'stronger' than others and some of these are present on the surfaces of erythrocytes as haemagglutinogens, they have been the most extensively studied and many alleles or pseudo-alleles are known at this locus (Amos, Gorer & Mikulska, 1955; Gorer, 1956; Snell, 1958; Gorer & Mikulska, 1959).

On appropriate immunization of mice of one inbred strain with the products of another, the subsequent graft of a tumour specific for the first strain can lead to enhancement of the growth of the tumour in a recipient which would otherwise reject such a tumour (Kaliss, 1958; Snell, Winn, Stimpfling & Parker, 1960). The distribution of histocompatibility (H) haemagglutinogens and of enhancing (E) antigens is the same as that of the T antigens, which give, under appropriate circumstances, hastened rejection of, for example, skin grafts. This leads to the view, now gaining acceptance, that T, E and H antigens are either the same substance or at least are closely associated in some way. Certain differences between the properties of