Oestrogen Conjugates of Human Late-Pregnancy Urine

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1. The separation of the oestrogen conjugates in late-pregnancy urine into two groups, peaks I and II, by gel filtration on Sephadex G-25 (Beling, 1963) has been shown to be affected by the presence of urate, which delays the elution of peak II conjugates. 2. By reapplication to a Sephadex column, peak I conjugates have been further separated into two groups (peaks IA and IB) and the metabolites in urine effecting this separation have been studied. 3. Further analysis of the mixed conjugates in the main groups IA, IB and II by ion-exchange and partition chromatography has led to the identification of some of the conjugates present. 4. Oestriol 3-sulphate 16α-glucuronide and 16α-hydroxyoestrone 3-sulphate 16α-glucuronide have been identified in peak IA. 5. The main components of peak IB have been identified as oestrone 3-glucuronide and oestriol 3-glucuronide. 6. The major conjugate in peak II was oestriol 16α-glucuronide and no oestriol 17β-glucuronide was found; small amounts of the ring-D monoglucuronides oestradiol 17β-glucuronide, 16-epioestriol 16β-glucuronide and 16α-hydroxyoestrone 16α-glucuronide were found in this fraction. 7. The behaviour of synthetic oestrogen monoglucuronides has been used as a guide in separation.

Oestriol glucuronide, the main oestrogen conjugate of late-pregnancy urine, was first isolated by Cohen & Marrian (1936). In subsequent studies (Cohen, Marrian & Odell, 1936; Grant & Marrian, 1950) the glucuronide was crystallized as the sodium salt and shown to correspond in composition to an oestriol monoglucuronide with a free phenolic group at the steroid C-3 position. The absolute structure of this conjugate was established by Neeman & Hashimoto (1962) by the conversion of the glucuronide into the totally methylated derivative and the subsequent hydrolysis of the latter. The identification of the resulting oestriol dimethyl ether as 3,17β-dimethoxyoestra-1,3,5(10)-trien-16α-ol established the constitution of the urinary compound as oestriol 16α-glucuronide, i.e. [3,17β-dihydroxyoestra-1,3,5(10)-trien-16α-yl β-D-glucopyranosid]uronic acid. Carpenter & Kellie (1962) converted the oestriol glucuronide fraction of a late-pregnancy urine by methylation and subsequent acetylation into the corresponding oestriol glucuronide triacetate methyl esters. Partition chromatography of the product on Celite and subsequent fractional crystallization yielded two crystalline compounds, which were identified as methyl [17β-acetoxy-3-methoxyoestra-1,3,5(10)-trien-16α-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]-uronate and methyl [16α-acetoxy-3-methoxyoestra-1,3,5(10)-trien-17β-yl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosid]uronate. These compounds correspond respectively to oestriol 16α-glucuronide and oestriol 17β-glucuronide.

Because of doubt expressed about the presence of oestriol 17β-glucuronide in late-pregnancy urine (Hashimoto & Neeman, 1963) and because the compound characterized as the 16α-glucuronide had been extensively purified before methylation, additional attempts have now been made to isolate the individual oestrogen conjugates of pregnancy urine, without derivative formation, by physical methods.

The advantage of Sephadex-gel filtration for the preliminary purification of the oestrogen conjugates of pregnancy urine has been demonstrated by Beling (1963), who showed that when urine is poured directly on to a Sephadex column the oestrogen conjugates are separated into two groups. Fractions containing the two groups, referred to as peaks I and II, can be eluted from the column by distilled water and the column can be re-used. Study of the behaviour of synthetic oestrogen conjugates suggests that oestrogen 3-glucuronides are eluted in peak I, and Beling (1963) has demonstrated the presence of oestriol 3-glucuronide and oestrone 3-glucuronide in this fraction. Peak II, which is less contaminated than peak I, contains oestriol 16α(?17β)-glucuronide as a major component.

In the present study, the analysis of the oestrogen conjugates of late-pregnancy urine has been carried out by Sephadex-gel filtration, by ion-exchange

chromatography on ECTEOLA-cellulose powder (Whatman ET11), and subsequently by partition chromatography on Celite and paper. A preliminary account of this work has been published (Smith & Kellie, 1965).

METHODS

The following trivial names have been used throughout the text and Tables: oestrone [3-hydroxyoestra-1,3,5(10)-trien-17-one]; oestradiol [oestra-1,3,5(10)-triene-3,17β-diol]; oestriol [oestra-1,3,5(10)-triene-3,16α,17β-triol]; epioestriol [oestra-1,3,5(10)-triene-3,16β,17β-triol]; 16α-hydroxyoestrone [3,16α-dihydroxyoestra-1,3,5(10)-trien-17-one].

The synthetic oestrogen monoglucuronides used were prepared as described by Elce, Carpenter & Kellie (1967).

Chromatography

Sephadex-gel filtration. Columns of two sizes were used, packed with Sephadex G-25 (medium grade) as described by Beling (1963). The larger preparative column (75cm. $\times$4·8cm. diam.) contained approx. 1·5l. of gel and was used on a preparative scale for urine and urine concentrates, and for the refiltration of large eluates from the primary column. The smaller column (52cm.$\times$1cm. diam.; 37ml. of gel) was used in an exploratory manner to study the behaviour of eluates from the larger column before processing the bulk of the material. With both columns distilled water was used as the eluent.

Ion-exchange chromatography. ECTEOLA-cellulose powder (Whatman ET11) was pre-cycled with washes of 0·5N-HCl and 0·5N-NaOH (*Whatman Technical Bulletin*, p. 5) and transferred to a column (50cm.$\times$1cm. diam.) in appropriate buffer. The material chromatographed was introduced in buffer solution and eluted with suitable inorganic solutions.

Partition chromatography. Celite partition columns (36–37cm.$\times$2cm. diam.) were prepared by adding stationary phase (0·6ml./g.) to acid-washed Celite 535 (50g.) and packing this in mobile phase, by using a Martin packer. The following biphasic systems (Bush, 1957) were used: ED_1, 2-methylpropan-2-ol–ethylene dichloride–acetic acid–water (3:17:6:14, by vol.); ED_2, 2-methylpropan-2-ol–ethylene dichloride–acetic acid–water (5:15:6:14, by vol.); T, 2-methylpropan-2-ol–toluene–acetic acid–water (5:15:6:14, by vol.).

Paper chromatography. Whatman no. 2 paper was used for descending chromatograms at 25°. For conjugates, the systems ED_2 (after tank equilibration for 24hr.) and BA (butan-1-ol–2-methylpropan-2-ol–2N-ammonia; 3:1:4, by vol.) were used. Oestrogens were chromatographed on formamide-impregnated paper with the mobile phases C_1 (chloroform) and C_2 (chloroform–ethyl acetate; 5:1, v/v).

Thin-layer chromatography. The chromatograms were run on plates (20cm.$\times$20cm.) with a layer (0·3mm.) of silica gel (Kieselgel G; E. Merck A.-G., Darmstadt, Germany) after 30min. equilibration. For conjugates the following systems were used: E_1, ethyl acetate–ethanol–acetic acid (2:1:1, by vol.); E_2, ethyl acetate–ethanol–acetic acid (3:1:1, by vol.); E_3, ethyl acetate–ethanol–acetic acid (3:3:2, by vol.). For oestrogens, system A (ethyl acetate–cyclohexane–ethanol; 9:9:2, by vol.) and system D (chloroform–ethanol; 9:1, v/v) were used (Lisboa & Diczfalusy, 1962).

Location and measurement of oestrogen conjugates

Kober reagent. The concentration of oestrogens in eluates from gel filtration, ion-exchange and partition columns was followed by carrying out Kober reactions on small portions of serial fractions. The method was not modified although conjugates, and not free oestrogens, were measured, and the hydrolysis of the conjugates presumably occurred during the preliminary stage of the Kober reaction. The procedure for oestrone, oestradiol, oestriol and 16-epioestriol was carried as described by Nocke (1961); for 16α-hydroxyoestrone the procedure for 16-epioestriol was used but extinctions were measured at 480, 518 and 556mμ.

Folin–Ciocalteu reagent. Fractions from columns were examined by chromatography on paper and on silica-gel thin-layer plates, and oestrogen conjugates were located by the Folin–Ciocalteu phenol reagent (Mitchell, 1952). The reagent locates phenolic metabolites, but does not reveal oestrogens conjugated at the C-3 position. On paper chromatograms 3-glucuronides were detected by dipping the paper in a buffered β-glucuronidase solution (1000 units/ml. in 0·5M-sodium acetate buffer, pH4·0) and incubating for 2hr. at 40°. The paper was allowed to dry in air and the liberated oestrogens were detected by the phenol reagent.

Acridine. Acridine (1% in ethanol) was used to locate glucuronides as described by Nordmann & Nordmann (1960) for the detection of organic acids. On paper chromatograms, glucuronides (containing 10μg. of oestrogen) were located as brown spots in u.v. light on ED_2 chromatograms (acid) and as more pronounced dark spots in u.v. light on BA chromatograms (alkaline).

Naphtharesorcinol. Naphtharesorcinol (1% in 2N-HCl) was used to detect glucuronic acid on thin-layer chromatograms. The solution was sprayed on to the plate, which was then heated at 105° in a covered dish containing conc. HCl; blue spots appeared in about 15min. It was essential that the cover prevented leakage from the dish, otherwise a general brown colour appeared and no blue spots were formed.

Anisaldehyde–sulphuric acid. Anisaldehyde–H_2SO_4 was used to detect oestrogens and oestrogen conjugates on thin-layer chromatograms (Lisboa & Diczfalusy, 1962, 1963).

Determination of glucuronic acid. Glucuronic acid in solution was determined by the method of Mead, Smith & Williams (1958) with naphtharesorcinol.

Determination of steroid sulphates. Steroid sulphates were determined by the method of Roy (1956) with methylene blue.

Hydrolysis of conjugates

Glucuronides, in solution, were hydrolysed by a suspension of powder B prepared from the limpet (*Patella vulgata*) (Dodgson & Spencer, 1953) in 0·5M-sodium acetate buffer, pH4·0, usually containing KH_2PO_4 (0·1M) to inhibit the arylsulphatase present in the preparation. The β-glucuronidase concentration was 500 (or 2500) units/ml. and incubation was carried out at 38° for 18 (or 3) hr.

Aryl sulphates were hydrolysed by a suspension of Mylase P (ex *Aspergillus oryzae*; Koch–Light Laboratories Ltd., Colnbrook, Bucks.), at 2mg./ml., in 0·1M-sodium acetate buffer, pH6·2, at 38° for 24hr. (Levitz, Katz & Twombly, 1965).

The liberated oestrogens were extracted with ether, the ether was washed with $NaHCO_3$ (8%, w/v) and with water and dried over Na_2SO_4. After the ether had been distilled off the oestrogens in the residue were identified by paper and thin-layer chromatography.

Solvolysis of oestrogen sulphates on paper chromatograms was carried out with dioxan and HCl as described by Schneider & Lewbart (1956).

RESULTS

Sephadex-gel filtration

Primary Sephadex column: formation of peaks I and II. A late-pregnancy (38th week) urine (24hr., 1990ml.) was filtered and divided into three equal volumes, which were passed independently through the large Sephadex column. Each portion of the urine was layered gently on to the column and allowed to percolate through (4·8ml./min.), and the eluate from the column during this period was rejected. When all the urine had entered the column, distilled water was added to the column from a constant head of approx. 20cm. of water. Development of the column could be followed by the formation and separation of two pigment bands, yellow and yellow–red, and the eluate from the column was collected automatically as serial fractions (80 × 20·5ml.). A typical separation of oestrogen conjugates into peak I (fractions 57–71, peak fraction 62), representing 44% of the total, and peak II (fractions 72–80, peak fraction 77), 56% of the total, is shown in Fig. 1; the values were obtained by carrying out Kober determinations on portions (0·05ml.) of each fraction of the eluate. Close correspondence in the separation of oestrogen conjugates was obtained in successive runs with the remaining portions of the urine, so that the combination of corresponding eluates could be carried out with confidence.

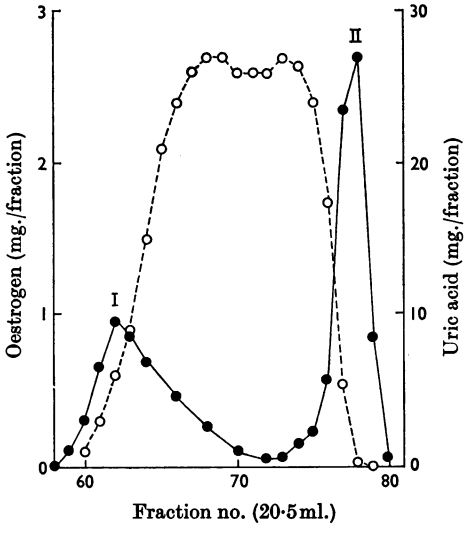

Fig. 1. Separation of oestrogen conjugates of late-pregnancy urine (BPU, 670ml.) into peak I (fractions 59–71) and peak II (fractions 74–79) by Sephadex G-25 column (75cm. × 4·8cm. diam.) chromatography. ●, Oestrogen estimated as oestriol by direct Kober reaction; ○, uric acid estimated by u.v. absorption.

Four urine samples obtained during the last 4 weeks of pregnancy from different subjects were processed; TPU (6-day collection), BPU (1-day collection), GPU (1-day collection, pregnancy resulted in twins) and LPU (1-day collection).

Small portions taken from the combined eluate of peak I and of peak II applied in normal male urine to the small column were re-eluted in their respective positions (I and II). Corresponding portions of peak I and peak II in 0·15M-sodium acetate buffer, pH6·5 (Beling, 1961), were both eluted in the peak I position. This behaviour implied that a factor present in normal urine was responsible for the separation of the two groups of oestrogen conjugates. Experiments with the components of urine showed that the factor which delays the elution of peak II is urate. Gel filtration of peak II conjugates in a solution of sodium urate (6mM) in 0·1M-potassium phosphate buffer, pH6·5, led to elution in the correct position and in this medium peak I and peak II conjugates could be separated. Elution of peak II conjugates was delayed by xanthine (1mg./ml.), but the effect was less marked; hypoxanthine, caffeine, adenine and all other urine components tested were without effect. Fractions from the primary column showed maximum u.v. absorption at 291mμ between the two oestrogen peaks; by using the extinction at this wavelength as a measure of urate concentration, a sharp fall in urate concentration, as peak II conjugates were eluted, was demonstrated (Fig. 1).

Behaviour of synthetic oestrogen monoglucuronides in the presence of urate. With the small Sephadex column (50cm. × 1cm. diam., 1ml. fractions) late-pregnancy urine gave peak I (fractions 35–39, peak fraction 36) and peak II (fractions 41–42). The synthetic glucuronides (containing approx. 50μg. of oestrogen), dissolved in 0·1M-phosphate buffer, pH6·5 (4·4ml.), containing 4mg. of uric acid, were applied to the column and were eluted with distilled water.

Oestradiol 3-glucuronide, oestradiol 17β-glucuronide, oestriol 16α-glucuronide and oestriol 17β-glucuronide were eluted in fractions 41–42 (peak II). Oestrone 3-glucuronide was eluted in

fractions 32–41 with two concentration maxima in fractions 32 and 39; oestriol 3-glucuronide was eluted in fractions 32–40 with maximum concentration in fraction 32. When these compounds were applied to the column dissolved in male urine they behaved in the same way, except for oestrone 3-glucuronide and oestriol 3-glucuronide, which showed maximum concentration at fractions 34–36 instead of fraction 32. 16-Epioestriol 16β-glucuronide behaved like the oestriol ring-D glucuronides, but 16α-hydroxyoestrone 16α-glucuronide was eluted in fractions 38–42 with maximum concentration in fraction 41 (Fig. 2).

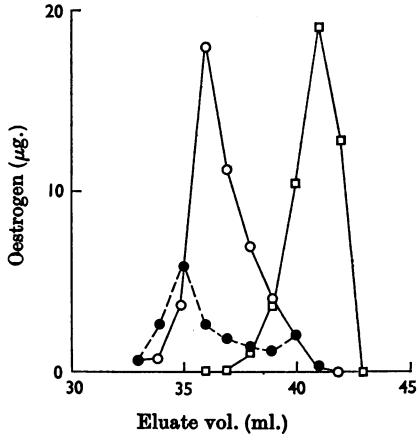

Fig. 2. Elution from a Sephadex G-25 column (52cm.× 0·9cm. diam.) of synthetic glucuronides dissolved in male urine (5ml.). ●, Oestrone 3-glucuronide; ○, oestriol 3-glucuronide; □, 16α-hydroxyoestrone 16α-glucuronide.

Subdivision of peak I urine conjugates on Sephadex: formation of peaks IA and IB. The oestrogen conjugates of peak I, which when reapplied to Sephadex in urate solution were eluted in the same fractions as from urine, behaved differently when dissolved in a minimum volume of 1·5M-acetate buffer, pH4·0, and applied to the same Sephadex column. The solution obtained by combining fractions under peak I from a 24hr. urine always contained urate. After adjustment to pH5·0 with acetic acid, uric acid was precipitated overnight at 4°. The uric acid was removed by filtration, the filtrate concentrated to about 20ml. and the pH readjusted to 4·0. Additional uric acid that was precipitated (1hr.) was filtered off and washed with water to give a combined filtrate volume of 30ml. This solution was applied to the large Sephadex column and the solutes were eluted with water (100 × 20·5ml.). Under these conditions the Kober-positive material from peak I was further subdivided into fraction IA (fractions 68–80, peak fraction 74), representing 20·5% of peak I, and fraction IB (fractions 85–105, peak fraction 95), representing 77·1% of peak I (Fig. 3). The corresponding maxima for the small Sephadex column were at fraction 39 (fraction IA) and fraction 52 (fraction IB).

The material applied to the Sephadex column (peak I) is an extremely complex mixture, and it has not been possible to characterize all the urine components that bring about the subdivision of oestrogen conjugates into fractions IA and IB. In addition to the oestrogen conjugates, the peak I eluate contains neutral steroid conjugates, e.g. pregnanediol, 17-oxo steroid and 17-hydroxy corticosteroid glucuronides, and many other

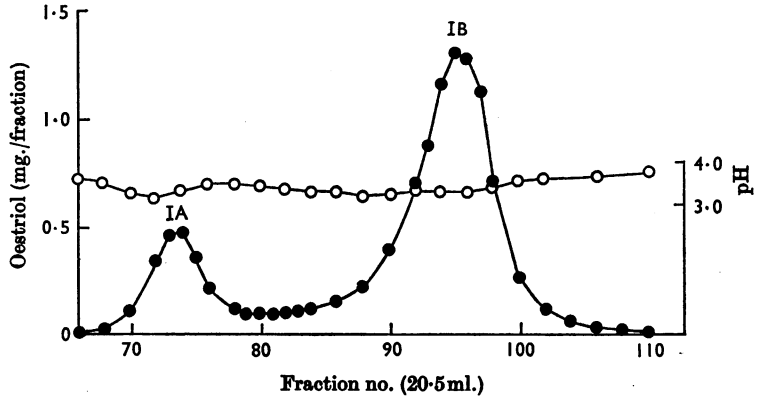

Fig. 3. Separation of oestrogen conjugates of peak I into peak IA (fractions 68–80) and peak IB (fractions 85–105) by Sephadex G-25 column (78cm.×4·8cm. diam.) chromatography. ●, Oestriol estimated by direct Kober reaction; ○, pH.

non-steroid metabolites. Examination by paper chromatography (Smith, 1960) of fractions from the Sephadex column in the region between peaks IA and IB has revealed the presence of normal urine components, some of which are acidic in character and effect the separation of peaks IA and IB. Compounds identified include indoxyl sulphate, *p*-hydroxyphenylacetic acid, *m*-hydroxyhippuric acid, *p*-hydroxyhippuric acid and indolylacetylglutamine. Of these, *p*-hydroxyphenylacetic acid and indoxyl sulphate, when added separately to artificial mixtures of fraction IA and fraction IB conjugates, delayed the elution of the fraction IB components from the small Sephadex column; moreover, they effected almost as complete a separation of peaks IA and IB as did the material eluted between peaks IA and IB from the large Sephadex column.

Behaviour of synthetic oestrogen monoglucuronides in the presence of p-*hydroxyphenylacetic acid.* On the small Sephadex column oestrone 3-glucuronide, oestradiol 3-glucuronide, oestriol 3-glucuronide, oestriol 16α-glucuronide, 16-epioestriol 16β-glucuronide and 16α-hydroxyoestrone 16α-glucuronide, when applied in 1·5M-acetate buffer, pH 4·0 (1ml.), were eluted by water in fractions 34–38. Addition of *p*-hydroxyphenylacetic acid (4mg.) to the acetate buffer resulted in the elution of all these compounds in fractions 47–51 (Fig. 4) and of peak IA material in fractions 35–38. Potassium indoxyl sulphate (4mg.) had a similar effect on some of the synthetic conjugates, but it produced a blue colour in the Kober reaction that interfered with the determination of the oestrogens; *p*-hydroxyphenylacetic acid had no effect on the Kober reaction.

Further chromatography of fractions IA and IB

Fraction IA. The extraction of the oestrogen conjugates from this column eluate presented some difficulty. Ethyl acetate extracted practically no Kober-positive material from a solution in 2N-hydrochloric acid; extraction of the conjugates by the ether–ethanol/ammonium sulphate procedure (Kellie & Wade, 1957) was incomplete unless the solution was initially brought to pH 2 and the extraction was carried out six times with 0·5vol. of ether–ethanol. The dried extract, weighing several hundreds of milligrams, contained less than 2mg. of 'oestriol' as measured by direct Kober reaction. It consisted of a brown gum containing crystalline material, and the oestrogen conjugates were stable when the solution was partially neutralized before evaporation. One sample of fraction IA (GPU) that had not been neutralized turned black overnight and had clearly changed in composition. A suspension of this residue in water behaved differently from fraction IA in that

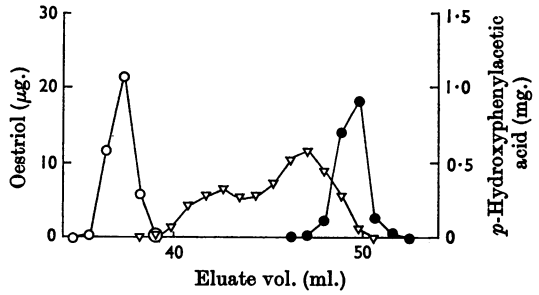

Fig. 4. Elution from a Sephadex G-25 column (52cm.× 0·9cm. diam.) of oestriol 16α-glucuronide: ○, dissolved in 1·5M-sodium acetate buffer, pH 4 (1·0ml.); ●, dissolved in 1·5M-sodium acetate buffer, pH 4·0 (1·0ml.), containing *p*-hydroxyphenylacetic acid (4mg.); ▽, *p*-hydroxyphenylacetic acid, estimated by u.v. absorption.

benzene extracted a purple–red pigment and left a brown colour in the aqueous layer; moreover, the oestrogens in the aqueous layer behaved as peak II on the small Sephadex column.

Further evidence of the instability of fraction IA (TPU) in acid solution was obtained after countercurrent distribution (20 tubes) in the system ethyl acetate–butan-1-ol–acetic acid–water (15:5:4:16, by vol.), whereby the oestrogen conjugates were divided into a more-polar and a less-polar fraction. By partition chromatography of the more-polar fraction in the same system, three components (IA_1, IA_2 and IA_3) were obtained. Component IA_1 (partition coefficient, K 0·10) and component IA_2 (K 0·24) gave, on hydrolysis with limpet powder B, in the absence of phosphate (β-glucuronidase and arylsulphatase), oestriol in 55% yield. Component IA_3 was found to contain oestriol 16α-glucuronide (39%), 16α-hydroxyoestrone 16α-glucuronide (26%) and other Kober-positive material of higher partition coefficient. The less-polar fraction was also found to contain oestriol 16α-glucuronide (67%) and 16α-hydroxyoestrone 16α-glucuronide (15%).

As neither of the identified monoglucuronides had been present in fraction IA it was clear that the nature of the conjugates had changed, possibly by solvolysis of sulphate esters in the acidic ethyl acetate partition system.

(*a*) Ion-exchange chromatography of fraction IA. DEAE-Sephadex was used by Hähnel (1965) to separate synthetic oestrone sulphate and oestriol 16α-glucuronide that had been added to male urine. The behaviour of the conjugated oestrogens of fraction IA on a column of ECTEOLA-cellulose, an ion-exchange material, was therefore studied.

After preliminary work, 319mg. of fraction IA

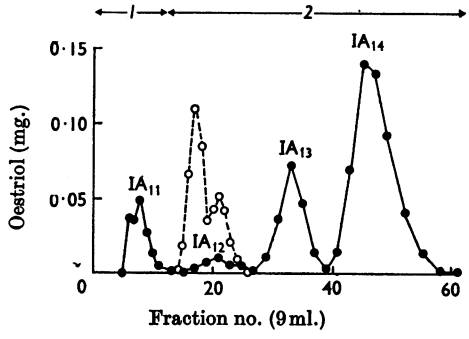

Fig. 5. Column chromatography on ECTEOLA-cellulose (50cm. × 1cm. diam.) of the oestrogen conjugates of peak IA with 0·1M-Na_2SO_4 (*1*) and 0·5M-Na_2SO_4 (*2*). ●, Peak IA (fractions 28–38, component IA_{13}, contained conjugated 16α-hydroxyoestrone; fractions 41–57, component IA_{14}, contained conjugated oestriol); ○, synthetic 16α-glucuronides of 16α-hydroxyoestrone (fractions 15–19) and oestriol (fractions 20–25).

containing 1·45mg. of 'oestriol' (BPU, nine-tenths of 24hr. sample) was applied to the ECTEOLA-cellulose column (50cm. × 1cm. diam.) in 0·1M-sodium sulphate and was eluted with 0·1M-sodium sulphate (100ml.) and 0·5M-sodium sulphate (600ml.). Kober determinations on the fractions (80 × 9ml.) showed separation into four components: IA_{11} (9·8%; fractions 5–12, peak fraction 8), IA_{12} (3·8%; fractions 13–27, peak fraction 21), IA_{13} (22·7%; fractions 28–38, peak fraction 33) and IA_{14} (63·7%; fractions 41–57, peak fraction 45) (Fig. 5). It is clear from Fig. 5 that fraction IA contained little if any oestriol 16α-glucuronide or 16α-hydroxyoestrone 16α-glucuronide, which, applied as the synthetic compounds, were eluted in the IA_{12} region.

The eluates IA_{13} and IA_{14} were freed from sodium sulphate by concentration to one-third volume and by the addition of ethanol (2·5vol.); the precipitated sulphate was filtered off and the filtrates were evaporated to dryness. To remove the remaining salt these residues were dissolved in 1·5M-sodium acetate buffer, pH4·0 (1ml.), and applied to the small Sephadex column. Fraction IA_{13} was eluted in fractions 36–43 to give a residue, weighing 9·8mg., containing 277μg. of '16α-hydroxyoestrone' by direct Kober reaction; fraction IA_{14}, eluted in fractions 37–44, gave a residue weighing 8·8mg. and containing 1·2mg. of 'oestriol'. Another sample of fraction IA (LPU), which weighed 2·1g. and contained 5·4mg. of 'oestriol' by direct Kober reaction, was treated similarly and gave an eluate residue IA_{13} weighing 9·6mg. and containing 363μg. of 16α-hydroxyoestrone-like material; the corresponding IA_{14} eluate residue weighed 16·3mg. and contained 2·9mg. of oestriol by direct Kober reaction. The eluates from the Sephadex columns were all acidic (pH4) and were adjusted to pH6·0 before evaporation. In each case a substantial improvement in oestrogen/fraction weight was achieved.

(*b*) Identification of components of fraction IA_{13}. Portions of fraction IA_{13}, containing the equivalent of 50μg. of oestrogen, from both BPU and LPU were examined by sequential treatment with sulphatase and β-glucuronidase. They were also examined by treatment in the reverse order, β-glucuronidase followed by sulphatase hydrolysis.

After incubation of fraction IA_{13} with sulphatase no Kober-positive compounds were extracted by ether. The conjugates extracted from this hydrolysate by ether–ethanol (Kellie & Wade, 1957) were chromatographed on paper in system ED_2 and gave several spots, one of which had the same mobility as 16α-hydroxyoestrone 16α-glucuronide. The extracted conjugates were then incubated with β-glucuronidase and an ether extract gave a 90% yield of free oestrogen measured as 16α-hydroxyoestrone. The free oestrogens from both samples, BPU and LPU, when chromatographed on paper in system C_1, gave two spots with the mobilities of 16α- and 16β-hydroxyoestrone.

When fraction IA_{13} was incubated with β-glucuronidase no Kober-positive material was extracted by ether. Conjugates extracted from the hydrolysate by the ether–ethanol procedure, when examined by paper chromatography in system ED_2, gave several spots of high polarity (10cm./48hr.), which were revealed, after dioxan–hydrochloric acid solvolysis, by Folin–Ciocalteu reagent. The conjugates were also resolved by thin-layer chromatography in system E_3 into a series of spots that did not correspond to 16α- or 16β-hydroxyoestrone 16-glucuronide. When the conjugates were further hydrolysed by sulphatase, ether extraction gave 42% (BPU) and 46% (LPU) of free oestrogen measured as 16α-hydroxyoestrone. Paper chromatography (system C_1) of this material showed that both samples contained 16α-hydroxyoestrone; that from the LPU sample also contained a compound with the same mobility as 16-oxooestradiol.

Determinations of glucuronic acid, by using naphtharesorcinol (Mead *et al.* 1958), gave the glucuronic acid/16α-hydroxyoestrone molar ratio as 1·75 in both samples of fraction IA_{13}. Steroid sulphate determinations, by using methylene blue (Roy, 1956), gave molar ratios 2·6 and 2·2. The findings are consistent with the presence of 16α-hydroxyoestrone 3-sulphate 16α-glucuronide in both samples of fraction IA_{13}.

(*c*) Identification of components of fraction IA_{14}. When portions of fraction IA_{14} (equivalent to 50μg.

of oestrogen) from BPU or from LPU were incubated with β-glucuronidase or with sulphatase, less than 1% of the Kober-positive material was extractable with ether. Thin-layer chromatography of fraction IA_{14} in system E_3 gave a zone that contained both oestriol and glucuronic acid but that had a mobility one-third of that of oestriol 16α-glucuronide.

The conjugate extract obtained after incubation with sulphatase contained oestriol 16α-glucuronide, as shown by paper chromatography in system ED_2 and by thin-layer chromatography in system E_3. Further hydrolysis of the extracted conjugates with β-glucuronidase gave oestriol, in quantitative yield, which was identified by paper and thin-layer chromatography (systems C_2 and A respectively).

Conjugated oestrogens extracted after the incubation of fraction IA_{14} with β-glucuronidase were examined by chromatography. Indoxyl sulphate was present in this extract: in the paper system ED_2 this compound moved slowly (3·6cm./48hr.), and was accompanied by a second chromatographic spot of mobility 5cm./48hr. that was detected with acridine, and with Folin–Ciocalteu reagent only after solvolysis. These compounds were also separated on paper in system BA (R_F values 0·37 and 0·51) and gave a positive test for ester sulphate with rhodizonic acid (Schneider & Lewbart, 1956). On thin-layer chromatography in system E_3, indoxyl sulphate was detected, together with one other chromatographic spot (R_F 0·53) that contained oestriol but that was different from oestriol 16α-glucuronide (R_F 0·45). Hydrolysis of the conjugate with sulphatase gave oestriol in yields of 100% (BPU) and 85% (LPU), identified by paper and thin-layer chromatography in systems C_2 and A respectively.

Determination of glucuronic acid in the two samples of fraction IA_{14} (BPU and LPU) gave glucuronic acid/oestriol molar ratios 1·66 and 1·38 respectively. Steroid sulphate determinations gave the sulphate/oestriol molar ratio as approx. 0·25 for fraction IA_{14} (BPU and LPU). In the conjugates extracted after β-glucuronidase hydrolysis of fraction IA_{14} (LPU) the sulphate/oestriol ratio was 0·75.

These results indicate that the main steroid component in eluate IA_{14} was oestriol 3-sulphate 16α-glucuronide.

Fraction IB. The oestrogen conjugates present in the Sephadex column eluate IB were extracted by the ether–ethanol/ammonium sulphate procedure and were separated on Celite in the system ED_2 into three components: IB_1, IB_2 and IB_3 (Table 1).

(*a*) Identification of component of fraction IB_1. The steroid component of fraction IB_1 was identified as oestrone 3-glucuronide by comparison with a synthetic standard on paper and thin-layer chromatograms. It gave a value of 1·1 for the glucuronic acid/oestrone molar ratio, and after hydrolysis with β-glucuronidase gave oestrone.

(*b*) Identification of the component of fraction IB_2. Fraction IB_2 gave a pink coloration with Kober reagent during the preliminary heating. Examination of the products of hydrolysis by β-glucuronidase indicated the presence of 16α-hydroxyoestrone, a compound known to give a pink colour during the early stages of the Kober reaction. Other products of the enzyme hydrolysis were present. The conjugate of fraction IB_2 had the same mobility on paper chromatograms as 16α-hydroxyoestrone 16α-glucuronide. A reference standard of 16α-hydroxyoestrone 3-glucuronide was not available.

(*c*) Identification of the component of fraction IB_3. The steroid component of fraction IB_3 was identified as oestriol 3-glucuronide by paper and thin-layer chromatography. In conjugate form it was detected by the acridine reagent and by Folin–Ciocalteu reagent after β-glucuronidase hydrolysis *in situ*. On paper chromatograms the conjugate and the reference compound did not move from the origin in system ED_2, but had R_F 0·48 in system BA and were clearly distinguishable from oestriol 16α-glucuronide (R_F 0·64) and from oestriol 17β-glucuronide (R_F 0·71) in this system. The conjugate gave a value of 0·9 for the

Table 1. *Oestrogen conjugates separated from eluate IB by chromatography on Celite columns (36cm. × 2cm. diam.) with system ED_2*

	Oestrogen [calc. as mg. of oestriol (Kober)]		
Late-pregnancy urine	IB_1	IB_2	IB_3
TPU	1·9	0·5	4·4
BPU	1·1	0·8	7·2
GPU	1·8	0·4	5·2
Main component	Oestrone 3-glucuronide	16α-Hydroxyoestrone 16α(?3)-glucuronide	Oestriol 3-glucuronide

glucuronic acid/oestriol molar ratio and after hydrolysis with β-glucuronidase gave oestriol. On thin-layer chromatograms run in system E_2, oestriol 3-glucuronide (R_F 0·29) was not effectively separated from oestriol 16α-glucuronide (R_F 0·25).

Chromatography of peak II conjugates

Behaviour of oestrogen ring-D monoglucuronides. Several synthetic oestrogen ring-D monoglucuronides, when added to male urine, were eluted from the Sephadex column in the peak II position; these included oestradiol 17β-glucuronide, 16α-hydroxyoestrone 16α-glucuronide, oestriol 16α-glucuronide, oestriol 17β-glucuronide and 16-epioestriol 16β-glucuronide. On Celite in system ED_1 oestradiol 17β-glucuronide (peak fraction 21) was clearly separated from the other $C_{18}O_3$ monoglucuronides (fractions 50–100). It is noteworthy that, in the same system, the isomeric forms of oestriol glucuronide, oestriol 16α-glucuronide (peak fraction 68) and oestriol 17β-glucuronide (peak fraction 82), could be separated when small amounts (equivalent to 200 μg. of oestriol) were used (Fig. 6). These two conjugates could also be separated by paper chromatography in system ED_2.

Preliminary separation of peak II conjugates. Although less contaminated than peak I with non-steroid urine components, peak II contained several oestrogen conjugates in small amounts mixed with a relatively large proportion of oestriol 16α-glucuronide. Conjugates extracted from the peak II eluate (TPU), containing the equivalent of 50 mg. of oestriol, when chromatographed on Celite in system ED_1 were incompletely separated (Fig. 7) and the maxima did not coincide with known ring-D oestrogen monoglucuronides. The elution maximum for oestriol 16α-glucuronide varied with the loading of the column. Rechromatography of selected fractions from this column was necessary.

(*a*) Oestradiol 17β-glucuronide. Fractions from the preliminary TPU Celite columns, corresponding to the elution position of synthetic oestradiol 17β-glucuronide (fractions 17–30 in Fig. 7), were combined and rechromatographed on a Celite column (36 cm. × 2 cm. diam.; 100 × 10 ml. fractions) in system T. Three Kober-positive components were found, one of which (fractions 45–62) had the same chromatographic mobility as synthetic oestradiol 17β-glucuronide (R_F 0·55) on paper in system ED_2. The residue from these fractions (27 mg.) contained the equivalent of 0·97 mg. of oestradiol. The glucuronic acid/oestradiol molar ratio was 1·09 and hydrolysis with β-glucuronidase gave an 85% yield of oestradiol by Kober reaction. The product of hydrolysis was identified by chromatography on paper (system C_1) and on thin-layer chromatograms (system A); Folin–Ciocalteu-positive compounds, other than oestradiol, were present in the enzyme hydrolysate. These results

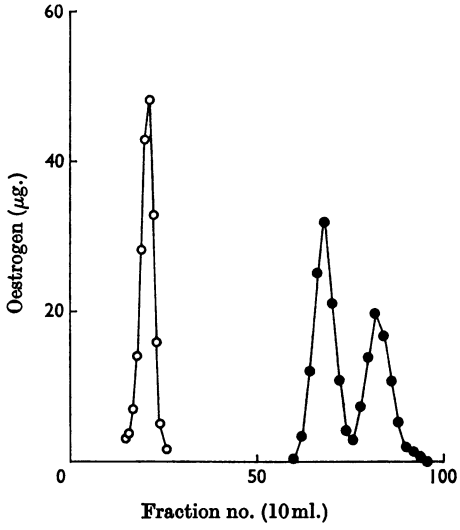

Fig. 6. Partition column chromatography on Celite (36 cm. × 2 cm. diam.) of synthetic oestradiol 17β-glucuronide (fractions 17–24) (○), oestriol 16α-glucuronide (fractions 62–74) and oestriol 17β-glucuronide (fractions 76–92) (●), with system ED_1.

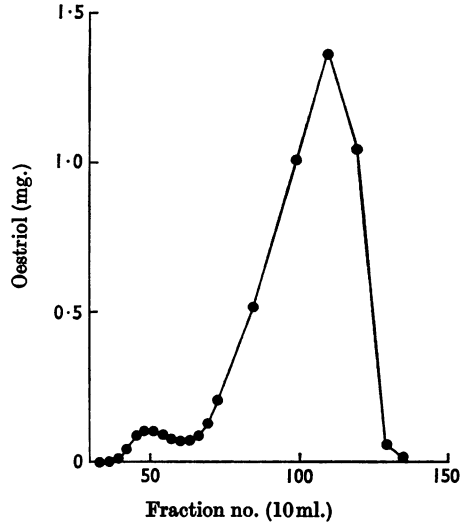

Fig. 7. Partition column chromatography on Celite (36 cm. × 2 cm. diam.) of oestrogen conjugates of peak II oestrogens, estimated as oestriol by direct Kober reaction, with system ED_1.

Table 2. *Thin-layer chromatography of eluate containing oestradiol 17β-glucuronide (fractions 17–30)*

Plate no.	Staining reagent	R_F value: Oestradiol 3-glucuronide	Oestradiol 17β-glucuronide	Eluate fractions 17–30
1	Anisaldehyde	—	—	0·21 (yellow fluorescence)
		0·42	0·47	0·47
		—	—	0·75
2	Naphtharesorcinol	0·42	0·47	0·47
		—	—	0·68
3	Folin–Ciocalteu reagent	—	0·49	0·49
		—	—	0·58
		—	—	0·81

indicate the presence of oestradiol 17β-glucuronide in the eluate.

Further evidence of the presence of other components was obtained by thin-layer chromatography of the unhydrolysed eluate (fractions 45–62) in system E_2. The results presented in Table 2 confirm the presence of oestradiol 17β-glucuronide, but also show that each of the three reagents used for locating the separated components, anisaldehyde, naphtharesorcinol or Folin–Ciocalteu reagent, revealed the presence of additional reacting compounds. These compounds and the minor Kober-positive components eluted from the Celite column were not identified.

(*b*) Oestriol 16α-glucuronide. The main oestrogen conjugate of peak II in all three urine samples BPU, LPU and TPU was identified as oestriol 16α-glucuronide by comparison on paper and thin-layer chromatograms with the synthetic compound. In partition column separations of the type illustrated in Fig. 7, it was the only oestrogen conjugate identified in fractions subsequent to fraction 75.

Fractions containing Kober-positive material eluted before fraction 80 were combined and submitted to repeated chromatography in the same system to give improved resolution of components (Fig. 8). The presence of 16α-hydroxyoestrone in fractions comprising the first peak (fractions 45–75 in Fig. 8) was indicated by the appearance of a pink coloration during the first stage of heating of the Kober reaction; moreover, hydrolysis of a portion of this material with β-glucuronidase liberated oestrogens that included 16α-hydroxyoestrone and 16-epioestriol. Fractions comprising the smaller peak of this column (fractions 79–99) yielded a residue of 35mg., which contained the equivalent of 2·2mg. of oestriol. The conjugate present gave a glucuronic acid/oestriol molar ratio 1·23 and behaved in all respects as oestriol 16α-glucuronide.

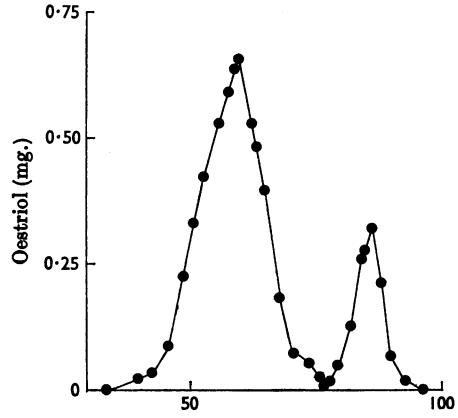

Fig. 8. Partition column chromatography on Celite (36cm. × 2cm. diam.) of the peak II components containing conjugated 16α-hydroxyoestrone, with system ED_1. Oestrogens were estimated with the oestriol Kober reagent. Some oestriol 16α-glucuronide (second peak) was separated.

(*c*) Oestriol 17β-glucuronide. Rechromatography of the material eluted in fractions 45–75 (Fig. 8) removed some, but not all, of the 16α-hydroxyoestrone 16α-glucuronide and 16-epioestriol 16β-glucuronide and gave a residue containing the equivalent of 1·45mg. of 'oestriol'. This material was eluted from the Celite column (system ED_1) in the same position as a reference sample of oestriol 17β-glucuronide and gave on paper chromatograms (system ED_2) an intense Folin–Ciocalteu-positive spot of the same mobility as the reference compound. The spot from the urine extract gave a blue fluorescence in u.v. light.

Further purification of the conjugates (equivalent to 1·45mg. of oestriol) was achieved by chromatography on a column (19·2cm. × 2cm.

diam.) of ECTEOLA-cellulose powder with 0·2M-sodium acetate buffer, pH 6·0, as mobile phase. The system not only separated the blue fluorescent compound from the oestriol glucuronides, but also separated the latter compounds from 16α-hydroxyoestrone 16α-glucuronide. Eluate fractions containing oestriol were concentrated to 0·2 vol. and cooled to 4°; concentrated hydrochloric acid (0·25 vol.) was added and the aqueous phase was extracted with ice-cold ethyl acetate (3 × 1 ml.). A portion (0·95) of the dry residue from the ethyl acetate extract was chromatographed on Whatman no. 2 paper in system ED_2 for 16 hr. alongside reference standards of oestriol 17β-glucuronide and 16-epioestriol 16β-glucuronide. As preliminary chromatograms (carried out on 0·05 of the dry residue) had shown the presence of a small proportion of Folin–Ciocalteu-positive material with the same chromatographic mobility as oestriol 17β-glucuronide, zones in the urine sample lanes corresponding to the reference compounds were cut out and eluted with aqueous ethanol (7:3, v/v).

Kober determinations carried out directly on these eluates were unsatisfactory. A portion (0·6) of the eluate corresponding to oestriol 17β-glucuronide, on hydrolysis with β-glucuronidase, liberated oestrogen that gave a normal Kober absorption equivalent to 19 μg. of oestriol in the whole eluate. Thin-layer chromatography of this material in system A showed that this oestrogen consisted mainly of 16-epioestriol with only a trace of oestriol. On the assumption that this trace was oestriol, that it constituted one-fifth of the oestrogen present and that it originated entirely from oestriol 17β-glucuronide, it was calculated that approx. 0·073 mg. of oestriol as the 17β-glucuronide had been separated from 121 mg. of oestriol conjugated as the 16α-glucuronide, i.e. an oestriol 16α-glucuronide/oestriol 17β-glucuronide ratio 1650.

Similar treatment of peak II conjugates from the urine samples BPU (384 mg. containing 21·1 mg. of oestriol) and GPU (193 mg. containing 23·3 mg. of oestriol) failed to give convincing evidence for the existence of more than trace amounts of oestriol 17β-glucuronide. The oestriol 16α-glucuronide/oestriol 17β-glucuronide ratio in these samples was not less than 350 and 430 respectively.

Although the separation of oestriol 16α-glucuronide and oestriol 17β-glucuronide can be achieved on Celite in the system ED_2 when the amounts of both are small (equivalent to 200 μg. of oestriol; Fig. 6), the resolution of these compounds is less satisfactory when the ratio of the two isomers is high as in urine. To study this problem, synthetic oestriol 17β-glucuronide equivalent to 0·2 mg. of oestriol was added to the oestriol 16α-glucuronide (equivalent to 10·5 mg. of oestriol) isolated from the BPU urine sample. Determinations of oestriol in the column fractions carried out by direct Kober reaction showed small but steadily increasing amounts of oestriol in fractions 64–75 and subsequently a rapid increase in oestriol to a maximum in fraction 86. No minimum concentration indicative of a separation of the isomers was found. Paper chromatograms in system ED_2 showed the presence of both conjugates in fractions 64–78 but only oestriol 16α-glucuronide in later fractions. The experiment showed that, when the ratio of the isomers is as low as 51, separation is incomplete and the detection of the minor component oestriol 17β-glucuronide is dependent on the absence of interfering substances.

(*d*) 16α-Hydroxyoestrone 16α-glucuronide. Fractions from the TPU peak II Celite and ECTEOLA-cellulose columns that contained 16α-hydroxyoestrone and 16-epioestriol were combined and rechromatographed on an ECTEOLA-cellulose column (42 cm. × 2 cm. diam.) with 0·2M-sodium acetate buffer, pH 5·8, as mobile phase. Kober determinations carried out on the eluted fractions (150 × 9 ml.) indicated the presence of 16α-hydroxyoestrone (5·4 mg.) in fractions 65–104 and 16-epioestriol (1·4 mg.) in fractions 108–144. The conjugates present were recovered by concentrating the eluates to 0·2 vol., cooling to 4°, acidifying with concentrated hydrochloric acid (0·2 vol.) and extracting with ice-cold ethyl acetate (3 × 1 vol.).

The extract from fractions 65–104 gave an almost colourless solid residue (33 mg.) that on paper chromatography in the system ED_2 gave a single Folin–Ciocalteu-positive spot of the same mobility as synthetic 16α-hydroxyoestrone 16α-glucuronide (R_F 0·29); the mobility was different from that of synthetic 16β-hydroxyoestrone 16β-glucuronide (R_F 0·34). Thin-layer chromatograms in the system E_3 gave a similar result and indicated the presence of glucuronic acid in the conjugate. A trace of glucuronide impurity was also present.

A portion of the ethyl acetate residue, after hydrolysis with β-glucuronidase, gave free oestrogens corresponding to 89% hydrolysis, and paper chromatography in system C_1 showed that the major component was 16α-hydroxyoestrone. A minor Folin–Ciocalteu-positive product of hydrolysis had a chromatographic mobility similar to that of 16β-hydroxyoestrone and different from that of 16-oxo-oestradiol.

(*e*) 16-Epioestriol 16β-glucuronide. The ethyl acetate extract of fractions 108–144 of the ECTEOLA-cellulose column gave a slightly coloured solid residue (6 mg.) that on paper chromatography in the system ED_2 gave a single Folin–Ciocalteu-positive spot corresponding to synthetic 16-epioestriol 16β-glucuronide (R_F 0·29) and distinct from synthetic 16-epioestriol 17β-glucuronide (R_F 0·235). Thin-layer chromatography

confirmed the presence of glucuronic acid in the conjugate.

After β-glucuronidase hydrolysis of the residue both paper and thin-layer chromatography confirmed the identity of the free oestrogen with a synthetic sample of 16-epioestriol.

DISCUSSION

In the light of present results, the use of Sephadex-gel filtration as a means of sorting out, in a preliminary way, the various types of urinary oestrogen conjugates is of considerable interest. It is a gentle process that is likely to cause little change of structure in the compounds present and is relatively simple to carry out. The mechanism of separation of oestrogen conjugates brought about by urate has not been explained; it does not appear to involve complex-formation as the oestrogen ring-D monoglucuronides were eluted after and not with the urate, nor can it be explained on the basis of molecular size in terms of the current theories of Sephadex action. The separation of conjugates is less precise than was originally thought, for, although most of the ring-D monoglucuronides were found exclusively in peak II, the elution of 16α-hydroxyoestrone 16α-glucuronide was not confined to this fraction. The ring-A monoglucuronides oestrone 3-glucuronide and oestriol 3-glucuronide were largely eluted in peak I, but 'tailed' into peak II, and oestradiol 3-glucuronide, somewhat unexpectedly, was eluted mainly in peak II.

The separation of peak I conjugates that occurred on Sephadex in the presence of *p*-hydroxyphenylacetic acid or indoxyl sulphate clearly resulted in the separation of the sulphate glucuronide diconjugates from the ring-A monoglucuronides, but again the mechanism is not understood. Although the effective metabolites in urine are both acidic, and the conjugates in peak IA are probably more highly ionized than those in peak IB, not all acidic compounds were effective, and Beling (1961) reported that oestrone 3-sulphate and the two oestradiol monosulphates were all eluted in peak II.

Oestriol 3-sulphate 16α-glucuronide, which has been identified as a component of peak IA, has been found in maternal blood during pregnancy (Touchstone, Greene, McElroy & Murawec, 1963). It has been detected in the urine of an anencephalic infant (Diczfalusy, Barr & Lind, 1964) and in mid-term pregnancy urine after the administration of [^{14}C]oestriol (Wilson, Eriksson & Diczfalusy, 1964). The second compound identified in this fraction, 16α-hydroxyoestrone 16α-glucuronide, is sufficiently unstable to impose restriction on the choice of solvent system that can be used for the resolution of fraction IA. Apart from the avoidance of acidic systems, which may result in solvolysis at C-3, alkaline systems are hazardous as they may produce epimerization at C-16α and lead to the formation of conjugates of 16β-hydroxyoestrone as artifacts (Elce *et al.* 1967). The isolation of 16α-hydroxyoestrone 3-sulphate 16α-glucuronide in the present study involved no alkaline conditions except possibly during ion-exchange chromatography. The compound was identified in two urines (BPU and LPU) and was probably present in the third. This double conjugate accounted for a considerable proportion of the 16α-hydroxyoestrone excreted and in one case (BPU) was 50% of the total.

Substantial amounts of oestriol were excreted as oestriol 3-glucuronide and in three of the urines examined the oestriol 3-glucuronide/oestriol 16α-glucuronide ratio was 0·33 (BPU), 0·22 (GPU) and 0·033 (TPU). These ratios support the conclusion of Goebelsman, Sjöberg, Wiqvist & Diczfalusy (1965) that oestriol 3-glucuronide is a major metabolite of oestriol.

In view of the previous claim (Carpenter & Kellie, 1962) to have demonstrated indirectly the presence of both oestriol 16β-glucuronide and oestriol 17β-glucuronide in late-pregnancy urine, considerable effort was made to isolate the latter compound from three such urines. Synthetic samples of these conjugates have been prepared (Elce *et al.* 1967) and it has been shown that they can be separated by partition chromatography on Celite and on paper. Although Kober-positive material was found with both media where oestriol 17β-glucuronide was expected, no convincing evidence of the presence of this conjugate was found.

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