The Analysis of Urinary 17-Oxo Steroids by Gradient Elution

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(*Received* 15 *October* 1956)

The present work was initiated by the observation of Reifenstein, Homburger & Dobriner (1950) that 3α:11β-dihydroxy-5β-androstan-17-one (11β-hydroxyaetiocholanolone), a minor component of the urinary 17-oxo steroids, was significantly associated with neoplastic growth. The re-examination of this problem has led to the development of a micro method for the quantitative separation and determination of the individual compounds which comprise the 17-oxo steroid fraction of urine.

Callow, Callow & Emmens (1938) first applied the Zimmermann (1936) reaction with *m*-dinitrobenzene and ethanolic potassium hydroxide for the determination and isolation of androgen metabolites from urine. Extension and development of this work led to the isolation and characterization of a large number of urinary steroids, many of which were 17-oxo steroids (Dobriner, Lieberman & Rhoads, 1948*a*; Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948; Lieberman, Fukushima & Dobriner, 1950). In contrast to the large-scale work of the American investigators, Dingemanse, Huis in't Veld & De Laat (1946) described a method for the analysis of the 17-oxo steroid fraction which could be applied to a urine sample smaller than a 24 hr. sample. The fraction was adsorbed on alumina and the individual compounds were eluted from the alumina with benzene containing increasing concentration of ethanol; the ethanol concentration was increased stepwise. This method in its original form and in the many modifications to which it gave rise (Robinson & Goulden, 1949; Zygmuntowicz, Wood, Christo & Talbot, 1951; Pond, 1951) has been of great value in the study of 17-oxo steroid metabolism.

The essentials of a reliable method for the analysis of the urinary 17-oxo steroid fraction are quantitative extraction, complete separation of compounds, unequivocal identification and precise determination. The existing methods do not fulfil these requirements and, in particular, are unsuited to the determination of the 11-hydroxy-17-oxo steroids. Three main improvements have been introduced.

The 17-oxo steroids are excreted almost exclusively in the conjugated form as glucuronides and as sulphates, and difficulty is experienced in bringing about the hydrolysis of these compounds. The traditional use of hot-acid hydrolysis is unsatisfactory as it introduces artifacts of dehydration and substitution and rapidly destroys the 11-hydroxy 17-oxo steroids. This difficulty has been overcome by enzyme hydrolysis, a preparation from the visceral hump of the limpet (*Patella vulgata*) being used as a source of β-glucuronidase (Dodgson & Spencer, 1953; Stitch & Halkerston, 1953). Although this preparation also contains a sulphatase capable of hydrolysing steroid sulphates, Roy (1956) has shown that this sulphatase is specific for compounds possessing the 3β-hydroxy-Δ^5 structure or the 3β-hydroxy-5α structure and does not hydrolyse 3α-hydroxy-5α-androstan-17-one sulphate (androsterone sulphate) or 3α-hydroxy-5β-androstan-17-one sulphate (aetiocholanolone sulphate), both of which are present in the 17-oxo steroid sulphate fraction of urine. For this reason enzyme hydrolysis of the sulphate conjugates has not been possible and the two kinds of conjugates have been hydrolysed consecutively, first the glucuronides by enzyme attack and subsequently the sulphates by adjusting the enzyme hydrolysate to pH 1 and extracting continuously with ether for 48 hr. (Lieberman, Mond & Smyles, 1954). The total 17-oxo steroid liberated by this combined procedure was at least as great as that obtained by hot-acid hydrolysis. Before hydrolysis the steroid conjugates were extracted from urine (Edwards, Kellie & Wade, 1953); this step not only eliminated much of the non-steroid matter present in urine, but, as the dry conjugate extract could be dissolved in a small volume of buffer, it enabled much higher concentrations of β-glucuronidase to be used.

Most workers have used adsorption chromatography on alumina to separate the individual 17-oxo steroids, and have eluted with benzene and subsequently introduced more hydrophilic solvents, in a stepwise manner, to elute the more tenaciously held compounds. Hagdahl, Williams & Tiselius (1952) have pointed out that only with compounds which have a linear-adsorption isotherm is it possible to develop a column in this stepwise manner satisfactorily; in the adsorption of 17-oxo steroids on alumina this is far from true, and the elution is always associated with 'tailing' which, in

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many cases, prevents the separation of closely related compounds. Lakshmanan & Lieberman (1953, 1954) applied the gradient-elution principle to the chromatographic separation of 17-oxo steroids on alumina and showed that it was possible to eliminate 'tailing' and achieve greatly improved separation. This gradient-elution principle has been used in the present method, but in place of the complex apparatus originally suggested a simple device has been introduced which enables the chromatographic separation to be achieved automatically. A further advantage of gradient elution is that it enables adsorbed compounds to be eluted from the column in a small volume of eluent.

A further adverse criticism of methods based upon the Dingemanse system is that the identity of the compounds is assumed according to their position in the chromatogram. Although it is true that with adequate precaution the elution of pure compounds from alumina-adsorption columns can be made with fair reproducibility, so that the main compounds of a normal urine may be tentatively identified in this way, interest rests chiefly on abnormal elution patterns where no certainty of identification exists. In the present method alternative methods have been suggested which enable the identity of compounds determined to be established. When sufficient material (100 μg.) was available the individual 17-oxo steroids were characterized by comparing the infrared-absorption spectra of the compounds eluted from the column with those of standard reference compounds (Dobriner *et al.* 1948*b*). When insufficient material was available for identification in this way, paper chromatography of the free 17-oxo steroids and the complexes which these compounds form with *m*-dinitrobenzene (Kellie & Smith, 1956) provided an alternative method of confirming identity and purity.

MATERIALS

β-Glucuronidase. This 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).

m-*Dinitrobenzene* (British Drug Houses Ltd., specially purified). This was recrystallized twice from ethanol and used as a 1% (w/v) solution in ethanol.

Ethanolic 2·5 N-KOH. This solution, prepared and used as recommended by Wilson (1954), may be stored for several weeks without significant rise in reagent blank value.

Other materials. Alumina (Savory and Moore Ltd., London): the water content was adjusted to 4·5%; *sodium acetate* (A.R.) buffer pH 4·0, 0·5 M; KH_2PO_4 and Na_2SO_4 anhyd. (A.R.); $(NH_4)_2SO_4$ (Laboratory reagent grade); *solvents*: ether, and dry benzene (laboratory-reagent grade) were distilled before use; *ethanol*: RR grade (James Burroughs Ltd., London); *Alundum chips* (Norton Abrasive Grain Co., Worcester, Mass.); *vials*: ½ dram (2 ml.) and 1 dram (4 ml.) metal-cap fitting (Johnsen and Jorgensen, London).

EXPERIMENTAL

Extraction of conjugated steroids from urine

The urine is collected without preservative and should be extracted while still fresh. Solid $(NH_4)_2SO_4$ is added to the urine sample (50 g./100 ml. of urine) and the mixture is shaken in a separating funnel until all solid has gone into solution. No adjustment of pH is necessary. The resulting solution is then extracted with 3:1 ether–ethanol mixture ($3 \times \frac{1}{2}$ of the original urine volume); it is wise to allow a clean separation of the liquid phases at each stage to promote rapid filtration of the extracts and to avoid the accumulation of aqueous phase in the combined extracts. When emulsions are encountered these can usually be broken by running off the aqueous layer and reshaking the emulsion vigorously or by increasing the volume of the ether–ethanol mixture to 1 vol. for each extraction. The combined ether–ethanol extracts after filtration (Whatman no. 1) are freed, if necessary, from traces of aqueous phase and are evaporated to dryness under reduced pressure (1 cm. Hg) and below 40°. A few pieces of Alundum greatly facilitate distillation, and whilst ether and ether–ethanol mixtures are being received distillation is rapid. Towards the end of the process distillation slows down because of the increasing water content of the distilled liquid. At this stage the receiver is emptied and ethanol is added to the distilling flask. When distillation is complete a yellow–brown gum is obtained which should be free of crystalline $(NH_4)_2SO_4$. To ensure the removal of the last traces of this compound the residual dry gum is dissolved in absolute ethanol (approx. one-tenth of the original urine volume) and filtered into the vessel used for enzyme hydrolysis. The ethanol is removed below 40° or by evaporation under a jet of N_2. If correctly carried out this procedure gives a residue which in aqueous solution does not give a sulphate precipitate with $BaCl_2$. The reliability of this process for extracting 17-oxo steroids has been checked on many occasions. Determinations of 17-oxo steroids carried out on such extracts, with appropriate correction for background, gives values which are at least as great as those obtained by applying the same procedure to urine. Furthermore, the extracted urine contains no measurable amount of steroid. It is advantageous to extract the urine with as small a volume of ether–ethanol as is necessary to complete the extraction of 17-oxo steroid conjugates, as increase in the volume of the extracting solvent increases the amount of contaminating solid extracted. The weight of conjugate extracts obtained by progressively decreasing the volume (V_s) of ether–ethanol used to extract a fixed volume (V_u) of urine was as follows. $V_s = V_u$, 364 mg. (446 μg.); $V_s = \frac{3}{4} V_u$, 286 mg. (434 μg.); $V_s = \frac{1}{2} V_u$, 196 mg. (457 μg.); $V_s = \frac{1}{4} V_u$, 102 mg. (410 μg.). The figures in parentheses, which refer to the amounts of 17-oxo steroids extracted, show that extraction with ½ vol. of ether–ethanol is satisfactory. As the extract is obtained without adjustment of pH and without excessive rise of temperature the process may be suitable for the extraction of corticosteroid and other unstable conjugates.

Hydrolysis of 17*-oxo steroid glucuronides*

It is convenient to carry out the enzyme hydrolysis of the glucuronides and the subsequent extraction in stoppered tubes (B24 standard ground-glass joint) which will withstand centrifuging. The dried residue of conjugates (half of

24 hr. urine sample) is dissolved in 0·5 M-sodium acetate buffer (pH 4·0, 25 ml.) and KH_2PO_4 (200 mg.) added. The enzyme preparation powder *B* (100 mg.; 100 000 units) is homogenized in sodium acetate buffer (20 ml.) and added to the solution of conjugates, and the remaining enzyme solution washed in with a further portion of buffer (5 ml.). Penicillin G (80 000 units) is added and the hydrolysis is carried out at 40° for 16 hr. At higher enzyme concentrations (20 000 units/ml.) hydrolysis was virtually complete after 1 hr. (91–95%) and complete at 2 hr., but overnight hydrolysis with a β-glucuronidase concentration of 2000 units/ml. is used as a convenient procedure. After incubation the free steroids are extracted with benzene (2 × 20 ml., 1 × 10 ml.) and centrifuged at 2000 rev./min. for 2 min. to break the emulsion, and the benzene layer is removed by a Pasteur pipette. The combined benzene extracts are washed with N-NaOH (3 × 5 ml.) and with water until neutral. After drying over anhydrous Na_2SO_4 the benzene is removed and the fraction assayed for 17-oxo steroid content. This procedure hydrolyses the 17-oxo steroid glucuronides completely but leaves the sulphate conjugates intact.

Hydrolysis of 17-*oxo steroid sulphates*

During benzene extraction of the enzyme hydrolysate, the 17-oxo steroid sulphates remain in the aqueous phase. This layer is diluted to a convenient volume (100 ml.), the pH value is adjusted to 1·0 with HCl and the resulting solution is extracted continuously with ether in a liquid–liquid extractor for 72 hr. The efficiency of this process as a means of hydrolysing steroid sulphates has been examined with 3β-hydroxyandrost-5-en-17-one sulphate (dehydro-*epi*androsterone sulphate; 500 μg.) as a model compound. Recovery of the free 17-oxo steroid was as follows: 48 hr., 87·0%; 72 hr., 94·6%. The ether layer, which contains the hydrolysed 17-oxo steroids, is washed with N-NaOH (3 × 10 ml.) and with water until neutral. After drying over anhydrous Na_2SO_4 the ether is removed and the fraction assayed for 17-oxo steroid content.

Determination of 17-*oxo steroids*

In order to determine small amounts of 17-oxo steroids, the Zimmermann reaction as used by Callow *et al.* (1938) has been scaled down to cover two additional ranges. In these determinations the quality of the ethanolic KOH is of paramount importance and the reagent blank must be low.

One-quarter scale; 0–50 μg. *of* 17-*oxo steroid.* On this scale the reaction is conveniently carried out in screw-capped vials (4 ml.) to prevent evaporation. The material to be assayed is transferred to a vial, dissolved in 1% (w/v) *m*-dinitrobenzene (0·1 ml.) and ethanolic 2·5 N-KOH (0·05 ml.) is added. After mixing by rolling the tube, the contents are maintained at room temperature for 1 hr. and are then diluted with ethanol (2·5 ml.). These determinations are conveniently carried out in batches of ten, with a simultaneous reagent blank and standard (15 μg. of dehydro*epi*androsterone), and provide enough material to be read in a 1 cm. cell of a prism spectrometer (Unicam 600). Readings are made at 440, 520 and 600 mμ and a background correction is made as recommended by Allen (1950). Provided the ethanolic KOH blank is low and a standard is run simultaneously it has not been found necessary to control the ambient temperature.

One-twentieth scale; 0–10 μg. *of* 17-*oxo steroid.* This reaction should also be carried out in screw-capped vessels (2 ml.). The material to be assayed is transferred to a vial, dissolved in 0·02 ml. of 1% *m*-dinitrobenzene and 0·01 ml. of ethanolic KOH is added. A simultaneous blank and standard (5 μg.) determination is made and after 1 hr. the reaction mixture is diluted with ethanol (0·5 ml.). Determinations on this scale are not as precise as those on the quarter-scale method but they supply sufficient material to fill the micro cell (0·5 ml.) of a Spekker absorptiometer, a spectrum green filter (Ilford 604) being used, and are used after chromatography as a guide in grouping fractions for more precise determination.

Gradient-elution chromatography

The chromatographic separation of the hydrolysed 17-oxo steroids is carried out on an alumina-adsorption column with a gradient of ethanol in benzene. Experience has shown that the moisture content of the alumina is important and that for good separation it should be between 4 and 5%. This moisture content refers to the loss in weight on heating 2 g. of alumina in a shallow weighing bottle at 100° for 16 hr.; alumina which has a lower moisture content than this may be made suitable by pipetting the required volume of water directly on to the alumina exposed in shallow trays. If the alumina is then mixed intimately for 2 hr. and the moisture content confirmed it can be used directly. This simple procedure was found to be more convenient and satisfactory than the elaborate process of exposure to solutions with vapours of known relative humidity. Provided the flow rate through the column does not exceed 25 ml./hr. the particle size of the alumina does not appear to be critical.

The column (35 cm. × 0·6 cm. inside diameter) is fitted at the top with a B14 standard ground-glass joint and has a capillary outlet (6 cm. × 0·2 cm. inside diameter) drawn to a fine point. When being filled, the column is closed at the outlet and filled with benzene; a small plug of cotton wool is pushed into position at the shoulder of the capillary and the alumina (6 g.) poured into the benzene in the dry state and allowed to settle under gravity. The outlet of the tube is opened and the column tapped uniformly and gently until the alumina has settled (height of column, 21 cm.). The 17-oxo steroid gum is transferred to the alumina column in benzene (3 × 0·2 ml.), and when the material has been adsorbed the column is filled with benzene. 17-Oxo steroids are not eluted from alumina with benzene and it is possible to obtain a very narrow starting zone which does not move until the ethanol gradient reaches it. As the hydrolysis of the glucuronides and sulphates is achieved independently, the free 17-oxo steroids from each source may be analysed separately or after combination. Whichever procedure is adopted the amount of material placed on the column is restricted to a maximum of 1 mg. of total 17-oxo steroid as determined by the Zimmermann reaction.

The apparatus for applying the gradient to the column (Fig. 1) consists of two reservoirs, a donor and a recipient vessel connected by a capillary siphon which passes through a small hole blown in the shoulder of the recipient vessel. The column is fed from the recipient vessel, which initially contains 0·2% of ethanol in benzene but which receives progressive enrichment from the donor. The contents of the recipient vessel are stirred.

The gradient reservoirs are filled independently whilst the column is prepared. The recipient vessel consists of a cylindrical separating funnel of 120 ml. capacity (Quickfit and Quartz no. D 1/22) holding 110 ml. of benzene containing 0·2% of ethanol, and the donor, which is fixed to the side of the recipient vessel by adhesive tape, consists of a large boiling tube (17 cm. × 2·4–2·5 cm. internal diameter) holding 65 ml. of benzene containing 2% (v/v) of ethanol. Although the volumes quoted are not critical it is important that the levels in donor and recipient vessels are equal before being connected; if necessary the effective area of cross-section of the donor can be reduced by inserting glass rods. The capillary siphon connecting the two reservoirs (0·1 cm. internal diameter) is filled with liquid of the same composition as that in the recipient vessel and is inserted in position. This arrangement will not work satisfactorily, without modification, when the density of the donor liquid is greater than that of the recipient liquid.

The reservoirs are placed in position on the column and the apparatus is mounted over the fraction collector with the stirrer in position and, in order to minimize evaporation losses, with the fine outlet from the column inside the counterpoised siphon of the fraction collector. The stirrer should reach down as far as the outlet of the capillary and should be driven by a spark-free induction motor. The development of the chromatogram is started by opening the tap of the recipient vessel. The eluent from a column of this type (6 g. of alumina; 21 cm. × 0·6 cm. internal diameter) runs at 10–12 ml./hr. and development is completed overnight, producing 80–85 fractions (2 ml.). These are collected in flat-bottomed vials (4 ml.) which are placed in wooden trays and taken to dryness overnight in a steam oven (shelf temperature 60°). The contents of the vials are redissolved in ethanol (2 ml.) and small portions are removed from each (one-tenth from fractions containing the major compounds dehydro*epi*androsterone, androsterone and aetiocholanolone; one-quarter from all other fractions) for assay by one-twentieth-scale Zimmermann reaction.

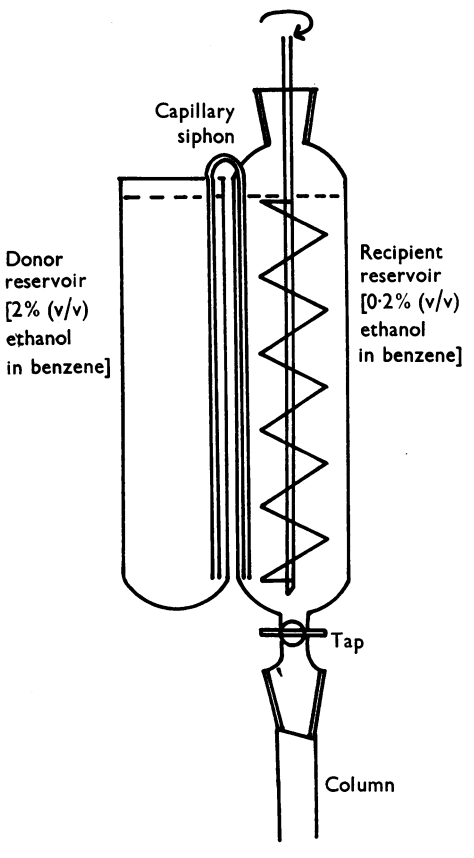


Fig. 1. Apparatus for separation of 17-oxo steroids by gradient elution.

Although requiring little labour this procedure takes some time, and for most clinical purposes a column of 3 g. of alumina (10 cm. × 0·6 cm. internal diameter) is satisfactory; the eluent from such columns runs at 20–25 ml./hr. and the development is complete in a working day. Furthermore, as little evaporation takes place, the small fractions to be taken from each vial may be removed as they become available and evaporated to dryness overnight with the main fractions. The only disadvantage of these smaller columns is that the two compounds 3α-hydroxy-5β-androstane-11:17-dione (11-oxoaetiocholanolone) and 3α:11β-dihydroxy-5α-androstan-17-one (11β-hydroxyandrosterone) are not completely separated from each other. At the end of each gradient chromatogram the column is eluted with 10% (v/v) of ethanol in benzene mixture (20 ml.) to remove small amounts of 17-oxo steroid still remaining on the column.

RESULTS

Fig. 2 has been constructed from the figures obtained by one-twentieth-scale Zimmermann reactions on the small portions removed from the main vials and serves as a guide for the bulking of the main fractions according to the clearly defined peaks. Provided the water content of the alumina remains unaltered and large stocks of donor and recipient solutions are made initially, the position of these peaks remains very constant, but cannot in all circumstances be relied upon as a means of identification. Again, although the figures obtained by one-twentieth-scale Zimmermann reaction give an indication of the amounts of various compounds present, the precise determination and the identification of the compounds is carried out on the remaining material.

The fractions combined according to the peaks of the graphs (Fig. 2) are again taken to dryness and redissolved in ethanol. Appropriate volumes (containing approx. 10 μg.) are taken from each sample and these are assayed by one-quarter-scale Zimmermann reaction, the absorption being read at three wavelengths, 440, 520 and 600 mμ, and an Allen correction applied. Fractions are removed from the same solutions for paper chromatography.

Identification of compounds

Source of standard compounds. No difficulty was experienced in obtaining synthetic specimens of dehydro*epi*androsterone, androsterone and aetiocholanolone. The

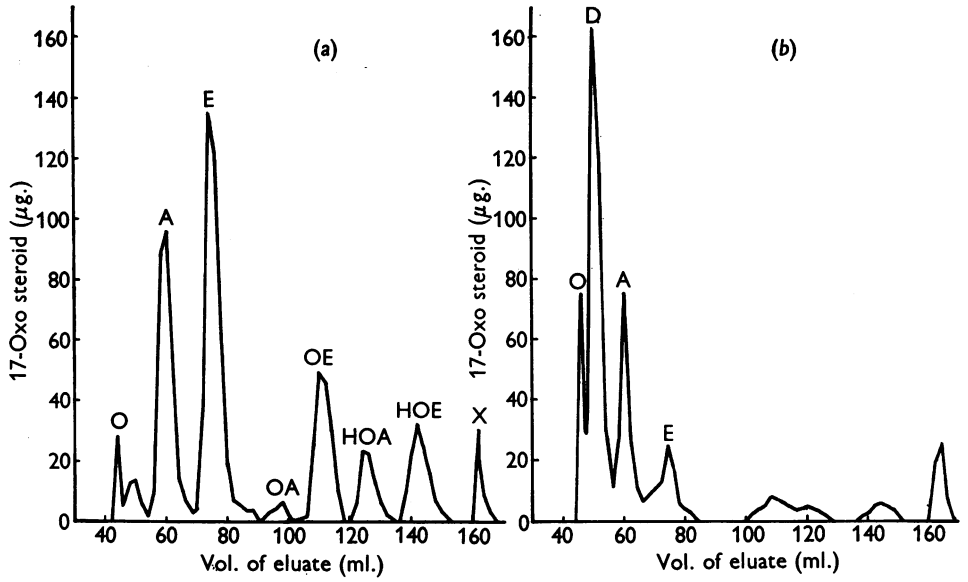
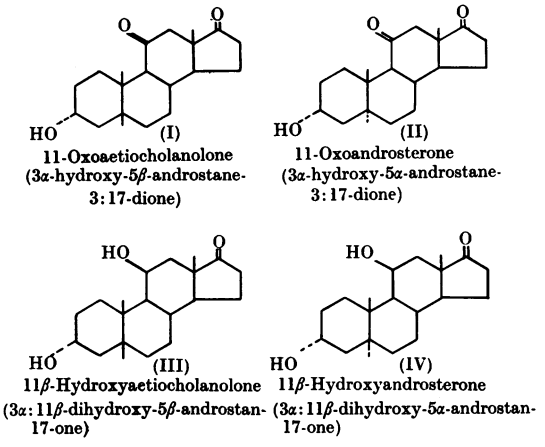


Fig. 2. 17-Oxo steroid excretion patterns of urine from a normal male subject. (*a*) Chromatogram of glucuronide fraction; (*b*) chromatogram of sulphate fraction. D, Dehydro*epi*androsterone; A, androsterone; E, aetiocholanolone; OA, 11-oxoandrosterone; OE, 11-oxoaetiocholanolone; HOA, 11β-hydroxyandrosterone; HOE 11β-hydroxyaetiocholanolone; O, a mixture containing androstane-3:17-dione and aetiocholane-3:17-dione; X, unknown.



starting material for the preparation of 11-oxygenated-17-oxo steroids was urine from two cases of adrenal tumour and from patients receiving cortisone therapy. In the latter instance the cortisone metabolites ('17-oxogenic' steroids) appearing in the urine were oxidized by sodium bismuthate (Norymberski, 1952) to the corresponding 17-oxo steroids. Steroid gums from these sources after conventional extraction and purification stages gave a ketonic fraction relatively rich in 11-oxygenated-17-oxo steroids which, by gradient-elution chromatography on alumina, yielded milligram amounts of 11-oxoaetiocholanolone (I); 11β-hydroxyandrosterone (IV) and 11β-hydroxyaetiocholanolone (III). These compounds, identified by infrared-absorption spectroscopy against reference samples, were used as standards in the subsequent methods of identification.

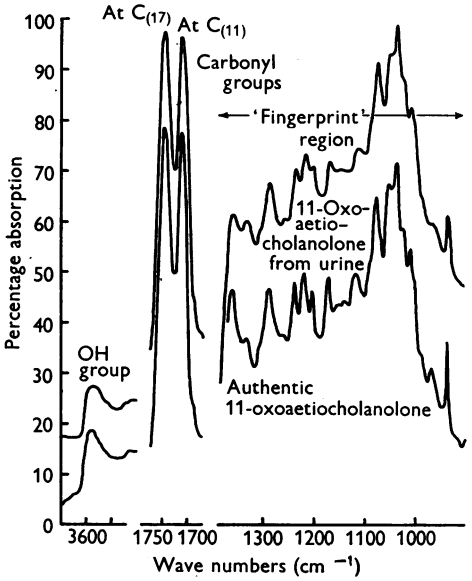


Fig. 3. Infrared-absorption spectra of 11-oxoaetiocholanolone. Upper curves, 11-oxoaetiocholanolone from urine; lower curves, 11-oxoaetiocholanolone (authentic specimen).

Methods of identification. When the amount of material permitted, identification was made by infrared-absorption spectroscopy with a Perkin–Elmer double-beam spectrometer (model 21) with micro cells (3 mm. optical path, 0·06 ml. capacity) and compensation of solvent absorption by variable space cell. Fig. 3 exemplifies the comparison of

the infrared-absorption spectra with a reference sample of 11-oxoaetiocholanolone (200 μg.) and corresponding material from a typical urine chromatogram. The spectra show absorption due to the hydroxyl group, the ketonic groups at $C_{(11)}$ and $C_{(17)}$ and the characteristic 'fingerprint' region. No difficulty was experienced in establishing the identity of the 11-deoxy-17-oxo steroids but the relative insolubility of 11β-hydroxyandrosterone and 11β-hydroxyaetiocholanolone in CS_2 prevented direct comparison. The difficulty was overcome by forming the 3-acetate derivative in each case.

When analysing 1 mg. of total 17-oxo steroid or less there is insufficient material to characterize the minor components by infrared spectroscopy. Under these circumstances identity and purity can be confirmed by running small amounts of the combined sample (5–10 μg.) on paper chromatograms against standard reference compounds. The 11-deoxy-17-oxo steroids run satisfactorily on the Bush *A* system (Bush, 1952): dehydro*epi*androsterone, R_F 0·32; androsterone, R_F 0·56; aetiocholanolone, R_F 0·43. The 11-oxygenated 17-oxo steroids can be identified on the Bush B_1 system: 3α-hydroxy-5α-androstane-11:17-dione (11-oxoandrosterone, II), R_F 0·70; 11-oxoaetiocholanolone, R_F 0·64; 11β-hydroxyandrosterone, R_F 0·50; 11β-hydroxyaetiocholanolone, R_F 0·40. In these Bush systems benzene was substituted for toluene to permit rapid removal of solvents after the paper chromatogram had been developed; the compounds were revealed by dipping successively into 2% (w/v) *m*-dinitrobenzene and ethanolic 2·5N-KOH and drying in a current of warm air. When insufficient material was available for determination and independent identification, the Zimmermann complex, present in the solution used for the determination, was used to identify the compound measured (Kellie & Smith, 1956). The same Bush chromatograph systems which are used for the free 11-oxo steroids may be used to identify the Zimmermann complexes. Only ethanolic 2·5N-KOH is necessary to develop the colour of the complexes which have the following R_F values. System *A*: dehydro*epi*androsterone, R_F 0·23; androsterone, R_F 0·46; aetiocholanolone, R_F 0·33. System B_1: 11-oxoandrosterone, R_F 0·88; 11-oxoaetiocholanolone, R_F 0·83; 11β-hydroxyandrosterone, R_F 0·78; 11β-hydroxyaetiocholanolone, R_F 0·70.

Composition of the glucuronide fraction

The value of mild hydrolytic procedures and gradient elution for the analysis of 17-oxo steroids is evident from the clean separation of the individual compounds of the glucuronide fraction (Fig. 2). There is a noticeable absence of artifacts of dehydration and substitution, and the complete resolution of androsterone and aetiocholanolone, and particularly of the 11-oxygenated derivatives of these 17-oxo steroids, is a marked improvement on that obtained by previous workers with the Dingemanse alumina column in the absence of a gradient. A further advantage of the method described is the small volume of eluent (170 ml.) necessary to complete the development of the column; this is of practical importance as it eliminates the need to evaporate a large number of bulky fractions. Evaporation of the chromatogram fractions overnight frequently left dehydro*epi*androsterone, androsterone, aetiocholanolone and 11-oxoaetiocholanolone in crystalline form. More than 300 urinary 17-oxo steroid glucuronide fractions analysed have shown the same general pattern and, as all the compounds identified in this fraction have been characterized previously by large-scale isolation studies, the main advantage of the method is that it enables a quantitative analysis to be carried out on small urine samples.

There is little doubt that the composition of the glucuronide fraction is more complex than the graph (Fig. 2) indicates. Examination, by paper chromatography, of the small amount of Zimmermann-positive material eluted from the column in front of androsterone usually showed the presence of at least two compounds. The material adjacent to androsterone, which in early experiments was present in the glucuronide fraction in varying amounts, was readily identified as dehydro*epi*androsterone. Subsequently, by adding 0·03M-KH_2PO_4 to the β-glucuronidase enzyme digest as an inhibitor of limpet sulphatases, only trace amounts of dehydro*epi*androsterone were present and it is probable that in normal subjects little, if any, of this compound is conjugated with glucuronic acid. An incidental advantage of adding phosphate is that pigment formation is greatly reduced. The remaining 17-oxo steroid component from this region of the alumina chromatogram was eluted slightly in front of dehydro*epi*androsterone and was more easily isolated after the use of phosphate. On alumina and on paper chromatograms the material behaved as a single compound; in running properties it resembled the C_{19}-diones 5α-androstane-3:17-dione and 5β-androstane-3:17-dione, which are difficult to separate. Similar 17-oxo steroid material is present, in higher proportion, in the sulphate fraction.

The identity of androsterone and aetiocholanolone was established by infrared records which showed the strong absorption of the 17-oxo group (1740 cm.$^{-1}$) and gave the characteristic absorption in the 'fingerprint' region (1200–800 cm.$^{-1}$). Nevertheless, more than one normal urine has been encountered which yielded a crystalline specimen of androsterone which gave an additional weak band at 1674 cm.$^{-1}$, corresponding to trace amounts of an unidentified compound containing a Δ^4-3-ketone group. Infrared methods were also used to characterize the four 11-oxygenated 17-oxo steroids, although this was not normally possible on isolated urine samples. 11-Oxoandrosterone was impure when eluted from the column and as it is a minor component of all urines it was necessary to combine small samples from several urines for infrared examination. The accumulated material after rechromatography on alumina was still impure when examined by paper chromatography, but the

component which had the same R_F value (0·70) as an authentic specimen of 11-oxoandrosterone gave two peaks in the carbonyl region at 1740 and 1705 cm.$^{-1}$ (authentic compound 1745 and 1707 cm.$^{-1}$). The 'fingerprint' region of the compound isolated from urine was not identical with that of the reference compound. The presence of a small absorption peak at 1674 cm.$^{-1}$ (Δ^4-3-ketone) is further evidence that the material was still impure.

The compound 11β-hydroxyaetiocholanolone has been found in all the urines examined, and a comparison of the urinary excretion of this compound by normal subjects and patients with neoplastic disease will be published elsewhere.

The small amounts of Zimmermann-positive material finally eluted from the columns with ethanol in benzene (10%, v/v) have not been identified.

Composition of the sulphate fraction

The main compounds in this fraction were dehydro*epi*androsterone, androsterone and aetiocholanolone. The destructive effect of hot-acid hydrolysis was more marked on the sulphate fraction than on the complementary glucuronides. Whereas in the latter fraction destruction was largely confined to the dehydration of 11-hydroxy-17-oxo steroids to form $\Delta^{9:11}$ compounds, hot-acid hydrolysis of the sulphates not only brought about extensive dehydration and substitution which affected the three main components but there was also substantial loss of total 17-oxo steroids. This is shown in Fig. 4, which gives a comparison of the hydrolysis of a urine sulphate fraction by hot-acid hydrolysis (30% HCl, v/v, at 100° for 10 min.) and by continuous ether extraction at pH 1. The latter method was slow but the product was virtually free from the artifacts invariably associated with hot-acid conditions. As an alternative, steroid sulphates can be more rapidly hydrolysed at room temperature by a mixture of anhydrous dioxan and trichloroacetic acid (Cohen & Oneson, 1953). The free 17-oxo steroids obtained by this method were also relatively free of artifacts but it was difficult to ensure the dryness of reagents and urine fractions necessary for consistent results. It is clear that the use of hot-acid hydrolysis in any quantitative method is undesirable.

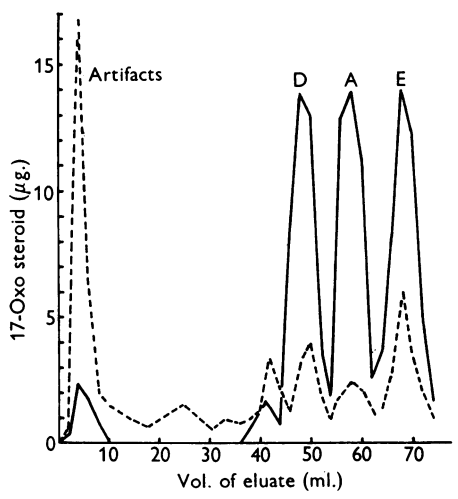


Fig. 4. A comparison of methods of hydrolysing 17-oxo steroid sulphates from urine. Chromatogram of 17-oxo steroids obtained by: - - - - -, hot-acid hydrolysis (30% HCl, v/v, at 100° for 10 min.); ——, continuous extraction with ether at pH 1. (D, Dehydro*epi*androsterone; A, androsterone; E, aetiocholanolone.)

The 17-oxo steroid eluted in front of the dehydro*epi*androsterone in the sulphate fraction was indistinguishable from the corresponding material in the glucuronide fraction. In behaviour on alumina and on paper chromatograms this material resembled the C_{19}-3:17-diones. Material (approx. 0·5 mg.) from several normal urines purified by rechromatography on alumina was resolved into three components on paper chromatograms with the Bush *A* system. The major component, which had the same R_F value as a reference standard of 5α-androstane-3:17-dione, when examined by infrared-spectrographic methods gave absorption peaks at 1739 and 1708 cm.$^{-1}$ in the carbonyl region. This is consistent with the C_3–C_{17}-dione structure of 5α-androstane-3:17-dione and its 5-β epimer, but in the 'fingerprint' region the record was not recognizable as either compound. The two minor compounds have not been identified.

The analysis of the sulphate fraction gave indifferent separation in that region of the chromatogram where the 11-oxygenated 17-oxo steroids normally appear. With sulphate fractions from normal urines the peaks of the alumina chromatograms were usually ill-defined, and in the subsequent paper chromatography only 11-oxoaetiocholanolone could be recognized. After the administration of cortisone, 11β-hydroxyaetiocholanolone has also been found in the sulphate fraction.

Applications of the method

The method has been in general laboratory use for three years and has been applied to many normal and pathological urines. In the latter, considerable variation in the composition of the glucuronide and sulphate fractions has been encountered without adverse effect on the separation of the individual steroid compounds. The simultaneous presence of other steroid metabolites does not appear to interfere and little, if anything, is gained by carrying out a Girard separation. It is important to stress that

the chromatograms (Fig. 2) are based, in each case, on the analysis of 1 mg. of 17-oxo steroid mixture and that they do not represent the absolute amount of the compounds present in the urine; for a comparison of the amounts of 17-oxo steroid excreted, whether in total or in the form of either conjugate, the amount of total 17-oxo steroid present as glucuronide and as sulphate must be taken into consideration.

The results shown in Table 1 were obtained by applying the method to a group of normal male subjects (six, 21–43 years old) and normal female subjects (six, 18–29 years old). Duplicate analyses carried out on one of the latter group showed good agreement. For identification and determination a minimum of 10 μg. of 17-oxo steroid from any individual chromatogram peak is necessary. Assuming that 1 mg. of total 17-oxo steroid can be obtained from one-tenth of a 24 hr. urine specimen, any compound excreted in amounts greater than 0·1 mg./day and resolved by the column can be determined. The accuracy of the determination is probably not better than $\pm 5\%$ for minor components. The figures of this small group of normal subjects shows the wide variations in excretion which occur within the normal range.

A selection of results obtained by analysis of urine samples from patients suffering from adrenal and other endocrine disorders (Table 2) illustrates the usefulness of the method for diagnostic purposes. A noteworthy feature of these results is the increased excretion of 11-hydroxy-17-oxo steroids in the patient with Cushing's syndrome. As previous methods of analysis based upon hot-acid hydrolysis would have destroyed these compounds, it is still uncertain whether this is a general finding or not. According to current ideas the 11β-hydroxy- and 11-oxo-aetiocholanolone arise from corticosteroid or other C_{21} steroid precursors (Dorfman, 1954), but the significance of the raised excretion of 11β-hydroxyandrosterone is at present unknown.

Table 1. *Analysis of 17-oxo steroid fractions from urine of normal subjects*

Figures are expressed as mg. equivalents of dehydro*epi*androsterone and are not corrected for variations in molecular extinction coefficient. P, Compounds, mainly C_{19}-diones, eluted before dehydro*epi*androsterone; D, dehydro*epi*androsterone; A, androsterone; E, aetiocholanolone; OA, 11-oxoandrosterone; OE, 11-oxoaetiocholanolone; HOA, 11β-hydroxyandrosterone; HOE, 11β-hydroxyaetiocholanolone. Unidentified material was eluted from the chromatograph column with 10% (v/v) ethanol in benzene. G, Conjugated as glucuronide; S, conjugated as sulphate.

				mg./24 hr.									
No.	Sex	Age	Fraction	P	D	A	E	OA	OE	HOA	HOE	Unidentified	Total
1	M.	21	G*	0·30	0·18	4·15	5·06	0·14	0·94	0·43	0·41	0·29	11·90
			S	Insufficient material									1·40
2	M.	25	G	0·28	0·35	4·54	4·54	0·10	0·64	0·46	0·15	0·21	11·27
			S	0·03	0·42	1·16	0·65		0·23			0·05	2·54
3	M.	28	G	0·02	0·38	1·72	4·34	0·10	0·52	0·36	0·20	0·19	7·83
			S	0·02	1·52	0·41	0·04		0·13			0·06	2·23
4	M.	29	G	0·03	0·37	3·25	3·10	0·07	0·48	0·41	0·26	0·14	8·11
			S	0·08	0·32	0·77	0·40		0·18			0·06	1·81
5	M.	36	G	0·43	0·50	1·75	2·97	0·13	0·77	0·40	0·50	0·20	7·65
			S	0·71	2·07	0·95	0·63		0·25			0·15	4·82
6	M.	43	G*	0·28	0·00	1·90	2·90	0·13	0·82	0·44	0·52	0·27	7·26
			S	0·36	0·25	0·34	0·21		0·25			0·09	1·50
7	F.	18	G	0·03	0·36	3·10	3·85	0·13	0·66	0·41	0·41	0·36	9·31
			S	0·13	1·40	0·83	0·45		0·27			0·11	3·19
8	F.	20	G*	0·18	0·24	2·39	4·48	1·26		0·58	0·43	0·35	9·91
			S	0·25	0·57	0·54	0·24		0·20			0·16	1·96
9	F.	25	G	0·24	0·80	2·87	3·57	0·10	0·78	0·45	0·38	0·31	9·50
			S	0·04	1·34	1·21	0·53		0·30			0·19	3·61
10	F.	26	G*	0·00	0·07	2·62	5·44	0·02	1·06	0·57	0·56	0·30	10·64
			S	0·17	0·97	0·72	0·76		0·22			0·04	2·88
11	F.	26	G*	0·07	0·27	3·23	4·66	0·16	1·35	0·23	0·09	0·27	10·33
			S	0·01	0·71	0·53	0·62		0·24			0·13	2·24
12	F.	29	G	0·07	0·38	2·38	5·22	0·09	0·55	0·16	0·33	0·23	9·41
			S	0·15	1·25	0·55	0·40		0·26			0·18	2·79

* KH_2PO_4 (0·03 M) present in enzyme digest as sulphatase inhibitor.

Table 2. *Analysis of 17-oxo steroid fractions from urine of patients with disorder of endocrine function*

Figures are expressed as mg. equivalents of dehydro*epi*androsterone and are not corrected for variations in molecular extinction coefficient. P, Compounds, mainly C_{19}-diones, eluted before dehydro*epi*androsterone; D, dehydro*epi*androsterone; A, androsterone; E, aetiocholanolone; OA, 11-oxoandrosterone; OE, 11-oxoaetiocholanolone; HOA, 11β-hydroxyandrosterone; HOE, 11β-hydroxyaetiocholanolone. Unidentified material was eluted from the chromatograph column with 10% (v/v) ethanol in benzene. G, Conjugated as glucuronide; S, conjugated as sulphate; T, total conjugates, glucuronide + sulphate.

					mg./24 hr.									
No.	Condition	Sex	Age	Fraction	P	D	A	E	OA	OE	HOA	HOE	Un-identified	Total
1	Adrenal tumour	F.	25	G*	0·29	1·67	4·85	14·50	0·95	1·04	1·20	1·58	1·70	27·78
				S	0·00	18·20	4·25	2·48			0·76		2·34	28·03
				T	0·29	19·87	9·11	16·98	—	—	—	—	4·04	55·81
	Post-unilateral adrenalectomy		—	T	0·10	0·16	0·15	0·24			0·18		0·07	0·90
2	Cushing's syndrome	F.	29	G*	0·53	0·18	1·33	2·94	0·37	1·35	2·51	1·76	0·65	11·62
				S	0·05	0·34	0·45	0·13			0·17		0·09	1·23
				T	0·58	0·52	1·78	3·07	—	—	—	—	0·74	12·85
	Post-adrenalectomy	F.	—	T	0·30	0·28	0·18	0·24	0·15	0·52	0·13	0·41	0·24	2·45
3	Adrenogenital syndrome	F.	30	G	0·20	0·94	1·00	4·20	0·10	0·69	0·36	0·12	0·17	7·78
				S	0·67	5·54	1·09	1·22			2·32		0·08	10·93
				T	0·87	6·48	2·09	5·42	—	—	—	—	0·25	18·71
4	Stein–Leventhal syndrome	F.	26	G	0·52	0·68	2·45	5·13	0·25	0·58	0·69	0·83	0·43	11·56
				S	0·24	1·46	0·77	1·04			0·33		0·16	4·00
				T	0·76	2·14	3·22	6·17	—	—	—	—	0·59	15·56
5	Arrhenoblastoma	F.	21	T	0·00	1·45	3·43	2·09	0·32	0·30	0·43	0·72	0·45	9·19
6	Carcinoma of breast after bilateral adrenalectomy + cortisone 37·5 mg./24 hr.	F.	29	T	0·00	0·32	0·14	0·22	0·50	2·56	0·25	0·65	0·31	4·45
7	Castrate	M.	34	T	0·00	0·56	0·25	0·55	0·06	0·29	0·24	0·14	0·08	1·67
8	Normal range (6 subjects)	F.	18–29	T	0·08–0·43	0·81–2·14	2·93–4·08	4·10–6·20	0·02–0·16	0·55–1·35	0·16–0·58	0·09–0·56	0·34–0·51	11·87–13·52

* KH_2PO_4 (0·03 M) present in enzyme digest as sulphatase inhibitor.

DISCUSSION

The method described provides a reliable procedure for the quantitative analysis of the 17-oxo steroid fraction of normal and pathological urines. Improved methods of hydrolysing steroid conjugates, which are an essential prerequisite for quantitative work, have been chosen to avoid the formation of artifacts and simplify the problem of chromatographic separation. The consecutive hydrolysis of glucuronides and sulphates, whilst increasing the time necessary for complete analysis, gives additional information about the form of conjugation of the 17-oxo steroids determined. The method is not intended to be a routine procedure but can be applied to daily urine specimens.

The improved chromatographic separation of closely related compounds is primarily due to the use of the gradient-elution principle. The failure of the Dingemanse system to separate, for example, the epimers androsterone and aetiocholanolone completely is due to the fact that in the system benzene–ethanol–alumina these compounds have curved adsorption isotherms and are eluted as unsymmetrical peaks with pronounced 'tailing' and overlapping. The slight gradient of ethanol in the benzene which constitutes the eluting solvent offsets this effect and the compounds are eluted as separated symmetrical peaks. It is important to realize that if 'tailing' is absent or has been suppressed and overlapping of symmetrical peaks still occurs, the gradient elution principle will not further aid separation. This is consistent with the theoretical treatment of this problem by Drake (1955).

Many factors influence the behaviour of the 17-oxo steroids adsorbed on an alumina column under gradient elution, and for this reason the procedure adopted is based upon an empirical study of the separation of an artificial mixture of those 17-oxo steroids occurring in the glucuronide fraction, under varying experimental conditions. Reference has been made to the effect of the moisture content of the alumina, but this is only one of several factors which can be varied to influence the movement and separation of compounds. These factors include the percentage of ethanol in the donor and recipient reservoirs, the dimensions of the reservoirs and the column and the flow rate. Of special interest is the ratio of the areas of cross-section of the reservoirs, for by varying this ratio the shape of the gradient curve may be made convex, linear or concave and suited to the particular requirements of the mixture to be analysed. Experimental treatment of the several effects of these variants upon the separation of 17-oxo steroid mixtures is beyond the scope of this paper, but such knowledge is essential for any radical change in the procedure.

The formation of a red pigment, presumably indigo red (Dobriner *et al.* 1948*a*), occasionally complicates the determination of 17-oxo steroids eluted from the alumina. This difficulty rarely arises in the analysis of the glucuronide fraction if phosphate is present during hydrolysis, and in the sulphate fraction the effect is restricted to a few fractions (1–3) immediately preceding the elution of dehydro*epi*androsterone, in which region the narrow band of pigment is eluted. The absorption spectrum of the pigment resembles that produced by the 17-oxo steroid Zimmermann reaction and absorption at 520 mμ cannot be neglected. When the amount of pigment is small, blank determinations carried out on additional portions of the chromatogram fractions without the addition of *m*-dinitrobenzene or ethanolic potassium hydroxide or both may be used to compensate for non-specific absorption; nevertheless, the safest procedure is to carry out a Girard separation on the total 17-oxo steroid fraction before chromatography. This difficulty does not arise with urines from healthy young people but has been encountered in urine samples from elderly normal subjects and from patients after surgical removal of endocrine glands. Usually in these circumstances the excretion of 17-oxo steroids is low and larger proportions of the daily output have to be processed.

SUMMARY

1. A new method for the quantitative analysis of the 17-oxo steroid fraction of urine has been described which avoids the formation of artifacts during hydrolysis of conjugates and which gives improved separation of individual 17-oxo steroids.

2. The hydrolysis of the 17-oxo steroid glucuronides has been achieved with β-glucuronidase prepared from limpets (*Patella vulgata*) and, subsequently, that of 17-oxo steroid sulphates by continuous extraction of an acidified aqueous solution with ether. The separate chromatographic analysis of material liberated by these processes has given information on the form of conjugation of individual 17-oxo steroids.

3. The application of the gradient-elution principle to the Dingemanse system for the chromatographic analysis of 17-oxo steroid mixtures has given improved separation, particularly of the 11-oxygenated 17-oxo steroids, all of which have been found in normal urine.

4. The identity of the separated 17-oxo steroids has been established by infrared spectroscopy and by the application of paper chromatography to the free 17-oxo steroids and to the complexes which these compounds form with *m*-dinitrobenzene.

5. Results obtained by the analysis of urine samples from a group of normal subjects and from

patients suffering from various forms of adrenal dysfunction are presented.

We are grateful to Dr T. F. Gallagher, Sloan Kettering Institute, New York, and to the Medical Research Council Steroid Reference Collection (Dr W. Klyne) for samples of the 11-oxo and 11-hydroxy-17-oxo steroids. We should also like to express our thanks to Sir Charles Dodds for his interest in this work.

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The Isolation of Oestrone from the Urine of Cows in Late Pregnancy

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(*Received* 15 *October* 1956)

The identification of the oestrogens secreted into the circulation of the cow, and also of those excreted, is of importance because it might lead to a greater understanding of the function of oestrogens in this species and in mammals generally. Since also it is known that different oestrogens are excreted by the human, the horse and the goat (Marrian, 1930; Marrian & Bauld, 1955; Girard, 1933; Klyne & Wright, 1956; Pincus & Thimann, 1955; Rodd, 1953), the interest in studying those of the cow is enhanced.

Pearlman, Rakoff, Cantarow & Paschkis (1947) have isolated oestrone, mainly present as the free phenol, from the gall-bladder bile of pregnant cows in amount approximating to 0·6 mg./l. Szego & Roberts (1946) and Pope & Roy (1953) have also reported oestrogenic activity in extracts of bovine pregnancy blood and colostrum respectively, in amounts equivalent to about 5 μg. of oestradiol-17β/l. in each. Cow's urine, particularly during late pregnancy, also yields oestrogenic extracts (Smith, Dickson & Erb, 1956). We have confirmed these findings in initial experiments with blood, colostrum and urine, and have found urine of late pregnancy to be much the richest source of oestrogen; this has therefore been investigated first.

METHODS AND RESULTS

Materials and bioassay

Materials. Diethyl ether was shaken with aqueous $FeSO_4$–H_2SO_4 solution to decompose peroxides (Vogel, 1948), although in the final stages of the work peroxide-free ether (May and Baker Ltd.) was used without any treatment. Toluene was a 'low-in-sulphur' grade (Hopkin and Williams Ltd.). Inorganic reagents were of analytical reagent quality. Celite 545 (Johns-Manville and Co. Ltd.) was used for chromatographic columns and was recovered for re-use by heating in air at 600°. Alumina was washed