A Method for the Quantitative Fractionation of Mixtures of 2-Methoxy-oestrone, Oestrone, Ring D α -Ketolic Oestrogens, Oestradiol-17 β , 16-*epi*Oestriol and Oestriol by Partition Chromatography and the Girard Reaction

By M. L. GIVNER,* W. S. BAULD† AND KITTY VAGI

Department of Metabolism and McGill University Clinic, Montreal General Hospital, Montreal, Canada

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The object of this investigation was to develop a method for the quantitative fractionation of pure 2-methoxyoestrone, oestrone, ring D α -ketolic oestrogens, oestradiol-17 β , 16-*epi*oestriol and oestriol. This required, first, a technique for the Girard reaction which would result in quantitative recoveries, and, secondly, a method whereby the ketonic and non-ketonic oestrogen residues could be separated into fractions by partition chromatography.

EXPERIMENTAL

Materials

Reagents used were A.R. unless otherwise stated. Diethyl ether, benzene, methanol, ethanol (96%, by wt.), Celite 535 and the Kober reagents were purified, prepared and tested as described by Bauld & Greenway (1957). The final distillation of solvents was done in all-glass apparatus.

Absolute ethanol. Purified ethanol (96%, by wt.) was left overnight with calcium oxide (300 g./l.), refluxed for 1 hr. and distilled under anhydrous conditions. Magnesium ethoxide was prepared with 75 ml. of dry ethanol, 5 g. of magnesium turnings and 0.5 g. of iodine. Dry ethanol (925 ml.) was then added, refluxed for 4 hr. and distilled (Vogel, 1951).

Hexane. Hexane (Anachemia, Montreal, Canada, technical grade, b.p. 65–69°) was washed with conc. H₂SO₄ (6 × 85 ml./l.) for 45 min. per washing, water (2 × 350 ml./l.), N-KMnO₄ in 3.7 N-H₂SO₄ (4 × 85 ml./l.) and left overnight with the last KMnO₄ portion. The hexane was then washed with water (4 × 170 ml./l.), 0.5 M-Na₂CO₃ (1 × 35 ml./l.), water (4 × 170 ml./l.) and dried over Na₂SO₄. The solvent (up to 3 l.) was poured through a column (48 in. × 1 in.) of 6–12-mesh silica gel, distilled and the 67.2–68.8° fraction collected.

Stained glassware was cleaned with a solution of 0.4 N-oxalic acid in 3.7 N-H₂SO₄.

Girard P reagent (Girard & Sandulesco, 1936). Commercial material was refluxed with acid-washed charcoal in ethanol (96%) for 15 min., filtered and left overnight at 5°. The crystals were washed with ethanol and stored in a brown bottle in a desiccator.

Asbestos. The material (Anachemia, Montreal, Canada, long fibre) was stirred with an excess of conc. HCl, washed with water until the washings were neutral and free of Cl⁻ and Fe³⁺ ions and dried at 140° for 24 hr.

Reference compounds. 16-*epi*Oestriol was obtained through the courtesy of Professor G. F. Marrian, 16-oxo-oestradiol-17 β from Professor Marrian and Dr M. Levitz and 2-methoxyoestrone from Dr T. F. Gallagher. 16 α -Hydroxyoestrone was prepared in collaboration with our colleagues Dr R. Greenway and Dr D. Douglas by a method based on that of Leeds, Fukushima & Gallagher (1954). The optical rotation of the prepared 16 α -hydroxyoestrone agreed with the value reported by Marrian, Loke, Watson & Panattoni (1957). As judged by column chromatography (for details see below) and paper chromatography (Givner, Bauld & Vagi, 1960), the eight steroids used in these studies were pure.

Fractionation and measurement of oestrogens

The final procedure (Fig. 1) is described below.

Separation of the ketonic from the non-ketonic oestrogens by the Girard reaction. The procedure for the Girard reaction was a modification of the method described by Pope, McNaughton & Jones (1957) and by Marrian, Watson & Panattoni (1957).

A mixture of about 10 μ g. of each oestrogen in ethanol was warmed and the solvent evaporated *in vacuo*. The residue was dried under CaCl₂ for 4 hr. in a vacuum desiccator. Absolute ethanol (1.26 ml.), Girard P reagent (0.126 g.) and acetic acid (0.25 ml.) were added to the flask. The reaction flask was left at room temperature (21 ± 3°) for 17 hr. and then cooled in a refrigerator (5°) for 1 hr. This was followed by the addition of 15 ml. of ice-water and sufficient 2.5 N-NaOH (cold reagent, approx. 1.5 ml.) to give a solution of pH 6. Three extractions were made with 20 ml. portions of ether, leaving the aqueous phase containing the ketonic fraction. The time for the separation of the two mixed phases was about 4 min. The ether extracts were combined and washed with 5 ml. of ice-water, and the aqueous phase was combined with the rest of the ketonic fraction. The ether extracts were washed with 2.5 ml. of m-NaHCO₃ (separation time of the phases, 7 min.), which was followed by a 5 ml. rinsing of the separating funnel, a wash (5 ml.) and a rinsing (20 ml.), all with water. The pH of the final rinsing was checked for neutrality (pH paper, range pH 2–10; Fisher Scientific Co., Montreal, Canada). The term 'wash' is used to describe the procedure that involved inversions of the separating funnel for the equilibration of the aqueous and organic phases;

* Fellow of the Salmon Foundation for Medical Research. Present address: Department of Endocrinology Southwest Foundation for Research and Education, San Antonio, Texas, U.S.A.

† Deceased.

Fig. 1. Flow sheet of the method for the separation of 2-methoxyoestrone, oestrone, 16 α -hydroxyoestrone (or 16-oxo-oestradiol-17 β), oestradiol-17 β , 16-epioestril and oestril.

a rinsing describes the addition of water for the removal of alkali from the inside walls of the separating funnel. The ether was distilled off, leaving the non-ketonic fraction. A second Girard reaction was carried out on the non-ketonic fraction to ensure removal of trace amounts of ketones not previously separated. The reaction was carried out by refluxing (side stem protected with CaCl_2) the non-ketonic fraction for 30 min. on a boiling-water bath with the same quantities of reagents as described above. After extraction of the non-ketonic fraction, the aqueous phase was discarded.

The aqueous (ketonic) fraction of the first Girard reaction was acidified to 1.2N with 7N- H_2SO_4 (approx. 4.5 ml.), left at room temperature for 2 hr. and extracted with ether (3 $\times$ 30 ml.). The ether was washed with 4 ml. of M- NaHCO_3 by 300 inversions of the separating funnel. This was followed by a rinsing (5 ml.), a wash (5 ml., pH checked for neutrality) and two rinsings (20 and 10 ml., after the final rinsing, pH was checked for neutrality), all with water. The extract was dried over anhydrous Na_2SO_4 and the ether distilled off, leaving the ketonic fraction.

Preparation of chromatographic column. Solvents were equilibrated and the chromatography was performed at a temperature of $18 \pm 0.5^\circ$. The separated aqueous (stationary) phase was added to dry Celite (5 ml./5 g.) and stirred for 3 min. An excess of organic (mobile) phase was added to the damp Celite and a uniform slurry was obtained by thorough mixing. A small amount of asbestos fibre (approx. 20 mg.) was inserted at the bottom of a chromatographic column (1 cm. $\times$ 15 cm.) to act as a filter plug to trap Celite particles that might have been eluted with the oestrogen. The column was supported with the lower end below the surface of a few millilitres of mobile phase in a test tube. Small portions of slurry were added to the top of the column and packed to the required height (10 or 12 cm.) by means of a plunger having a 0.71 mm. hole in the disk (Bauld, 1955). Sufficient pressure was exerted during the packing to give a solvent flow rate of 16–18 ml./hr.

Determination of column parameters. The periodic check of column parameters was necessary to make certain that no inert, mobile or stationary phase was lost during the packing of the columns and to ensure their reproducibility. The determination of column parameters was based on the work of Martin & Synge (1941) and Butt, Morris, Morris & Williams (1951). The equation of Butt *et al.* (1951) may be rearranged to give

$$V_r = KLA_s + LA_L \quad (1)$$

where V_r = the retention volume = volume of eluate to peak concentration of solute (ml.); K = partition coefficient = (concentration of solute in stationary phase)/(concentration of solute in mobile phase); L = length of column (cm.); A_s = cross-sectional area (cm.²) of column occupied by stationary phase; A_L = cross-sectional area (cm.²) of column occupied by mobile phase. For these determinations a slurry containing a known weight of Celite was packed in the column and the length of the column measured. The weight of the dry Celite (W_I) used and its density (D_I , 2.34) was used to calculate the volume of the inert phase (V_I , equation 2). From V_I and the length of the column, the cross-sectional area of the inert phase (A_I , equation 3) was calculated:

$$V_I = \frac{W_I}{D_I} \quad (2)$$

$$A_I = \frac{V_I}{L} \quad (3)$$

The cross-sectional area of the mobile phase (A_L) was determined from L and the retention volume of a dye (Sudan Red) with zero partition coefficient for the stationary phase. The dye was applied to the column in mobile phase and the eluate from the column collected until the dye appeared at its maximum concentration in the eluate. This volume was then the retention volume (V_r) of a solute of $K = 0$. From equation (1), when $K = 0$,

$$A_L = \frac{V_r}{L} \quad (4)$$

The cross-sectional area of the empty column (A cm.²) was determined from the volume of water occupied in a known length of column

$$A = \frac{V}{L} \quad (5)$$

The cross-sectional area of the stationary phase (A_s) was calculated by the difference between the cross-sectional area of the empty column and the sum of A_I and A_L :

$$A_s = A - (A_I + A_L) \quad (6)$$

The theoretical A_s was calculated from the volume of stationary phase (V_s) added to the Celite and the length of the column:

$$A_s = \frac{V_s}{L} \quad (7)$$

Table 1. Recoveries of oestrogens submitted to the Girard reaction by the final procedure

Oestrogen added	Amount added (μ g.)	No. of expts.	Reaction time (hr.)	Reaction temp.	Recovery (%) (non-ketonic)		Recovery (%) (ketonic)	
					Mean	Range	Mean	Range
2-Methoxyoestrone	9.8	8	17	21 $\pm$ 3°	0	0	99	95-100
Oestrone	10.1	8	17	21 $\pm$ 3	3	2-4	96	94-99
16 α -Hydroxyoestrone	11.9	4	17	21 $\pm$ 3	9	9-10	88	86-91
16-Oxo-oestradiol-17 β	10.0	4	17	21 $\pm$ 3	3	2-3	93	93-94
Oestradiol-17 β	10.0	19	17	21 $\pm$ 3	100	94-107	0	0
Oestradiol-17 β	10.0	4	0.5	130 $\pm$ 3	99	98-100	0	0
16- <i>epi</i> Oestriol	9.2	2	17	21 $\pm$ 3	100	100	0	0
16- <i>epi</i> Oestriol	9.2	4	0.5	130 $\pm$ 3	100	100	0	0
Oestriol	9.9	4	17	21 $\pm$ 3	100	98-100	0	0
Oestriol	9.9	8	0.5	130 $\pm$ 3	97	94-101	0	0

The column was packed from a slurry containing 5 ml. of stationary phase (equilibrated with mobile phase no. 1) and 5 g. of Celite; after stirring, excess of mobile phase no. 1 was added to the slurry. The Celite-column dimensions were 1 cm. $\times$ 10 cm. The extract was transferred to the column. Rinsing of the test tube containing the extract was done with mobile phase no. 1 from a separating funnel (containing 60 ml.), which was then fitted to the top of the column. The funnel had a stopper with an air leak. The eluate from 0 to 10 ml. was discarded and the next 50 ml. collected, giving the oestradiol-17 β fraction. With mobile phase no. 2, the 16-*epi*oestriol and oestriol fractions were eluted with 45 and 107 ml. respectively. The solvents were removed *in vacuo* by heating in a boiling-water bath.

Partition chromatography of the ketonic fraction. The ketonic oestrogen fraction was transferred to the Celite pad as previously described. The chromatogram was prepared with the following solvent system: stationary phase, methanol-water (70:30, v/v); mobile phase no. 1, hexane-benzene (98:2, v/v); mobile phase no. 2, hexane-benzene (50:50, v/v). The column was packed from a slurry containing 5 ml. of stationary phase (equilibrated with mobile phase no. 1) and 5 g. of Celite; after stirring, excess of mobile phase no. 1 was added to the slurry. The Celite column dimensions were 1 cm. $\times$ 12 cm. The extract was transferred to the column. Rinsing of the test tube which contained the extract was done with mobile phase no. 1 from a separating funnel (containing 52 ml.) having a stopper and air leak. The eluate from 0 to 10 ml. was discarded and the next 42 ml. was collected, giving the 2-methoxyoestrone fraction. With mobile phase no. 2, the oestrone and 16 α -hydroxyoestrone (or 16-oxo-oestradiol-17 β) fractions were eluted with 40 and 92 ml. respectively. The solvents were removed *in vacuo* by heating in a boiling-water bath.

Colorimetric estimation. The oestrogen residues were dissolved in ethanol and portions were transferred to test tubes (23 mm. $\times$ 150 mm.); quinol (25 $\pm$ 5 mg.) was added and the solvent evaporated in a boiling-water bath with an air stream. The Kober reaction was carried out by the procedure previously described for three oestrogens (Bauld, 1956). 'Oestradiol' reagent (3.3 ml.) was added to the oestradiol-17 β and 2-methoxyoestrone residues; 'oestrone' reagent (3.0 ml.) was added to the oestrone residue; 'oestriol' reagent (2.6 ml.) was added to the 16 α -hydroxyoestrone (or 16-oxo-oestradiol-17 β), 16-*epi*oestriol and oestriol residues. The tubes were then heated for 20 min. in a boiling-water bath (shaking after 5 and

10 min.), cooled in cold water and quinol was added (25 $\pm$ 5 mg.). Water (0.3 ml.) was added to the test tube containing oestrone and 0.7 ml. to those containing the 16 α -hydroxyoestrone (or 16-oxo-oestradiol-17 β), 16-*epi*oestriol and oestriol residues. The tubes were reheated for 15 min. (shaking after 5 and 10 min.), cooled in cold water and extinctions were measured against reagent blanks in a Beckman model B spectrophotometer. Readings were corrected for non-specific pigments by applying Allen correction equations (Allen, 1950). For oestrone, 16 α -hydroxyoestrone (or 16-oxo-oestradiol-17 β), 16-*epi*oestriol and oestriol the wavelengths were 480, 512.5 and 545 m μ ; for oestradiol-17 β they were 480, 515 and 550 m μ ; for 2-methoxyoestrone they were 512.5, 547.5 and 582.5 m μ . Appropriate standard curves were prepared. The amount of oestrogen in each fraction was calculated from its corrected extinction and that of the appropriate standard by simple proportion.

RESULTS

Experimental conditions and recoveries on seven oestrogens submitted to the Girard reaction

Many preliminary experiments were necessary to determine and establish the experimental conditions for the Girard reaction used in the final procedure. The recoveries by the final technique are shown in Table 1. The salient results of the preliminary studies on the individual oestrogens are described below.

2-Methoxyoestrone. The oestrogen was partly destroyed when in water (12% loss after 1 hr.), in 4N-HCl (21%) and in 4N-H₂SO₄ (26%). The destruction by the 4N-H₂SO₄ increased with time. However, the oestrogen was not affected by treatment with 1.2N-H₂SO₄ for 1 hr. but was partially destroyed by 1.2N-HCl (18%). The addition of 36N-H₂SO₄ (final solution was 1.2N) for the hydrolysis of the Girard complex partly destroyed the oestrogen (12-18%), but this was not true of 7N-H₂SO₄ (final solution was 1.2N). With 7N-H₂SO₄, 2 hr. was required for the complete hydrolysis of the Girard complex.

Oestrone. Experiments showed that 3% of the oestrogen may remain in the non-ketonic fraction.

16 α -Hydroxyoestrone. Studies showed that the 16 α -hydroxyoestrone found in the non-ketonic fraction was not decreased by an increase in the reaction time from 17 to 24 hr. or a decrease in the amount of acetic acid. A change in pH from 6 to 7 after neutralization resulted in no improvement. The use of aq. NH_3 soln. instead of NaOH diminished the amount of oestrogen in the non-ketonic fraction but no significant increase was apparent in the ketonic fraction. When a second Girard reaction was carried out on the ketonic fraction, a small percentage of the oestrogen was found once more in the non-ketonic fraction. The non-ketonic fraction from a Girard reaction on 119 μg . of 16 α -hydroxyoestrone was identified as 16 α -hydroxyoestrone by paper chromatography.

16-Oxo-oestradiol-17 β . Experiments showed that 93 % of this oestrogen was recovered in the ketonic fraction and 3 % in the non-ketonic fraction.

Oestradiol-17 β , 16-epioestrinol and oestrinol. These oestrogens were quantitatively recovered with either a long reaction time at room temperature or a short reaction time at a high temperature (130°). The use of absolute ethanol appeared to be important for quantitative recoveries of oestrinol.

Elution pattern of non-ketonic oestrogens

Fig. 3 shows the separation of oestradiol-17 β , 16-epioestrinol and oestrinol. In this experiment 28 ml. of eluate between the oestradiol-17 β and 16-epioestrinol fractions, and 64 ml. between the 16-epioestrinol and oestrinol fractions, contained no oestrogen. This system showed excellent reproducibility, as determined by the measurement of column parameters (Sudan Red was used as the dye with zero partition coefficient for the stationary phase). Typical column parameters were as follows: column length, 11.5 cm.; column area, 0.88 cm.²; inert-phase cross-sectional area, 0.15 cm.²; mobile-phase cross-sectional area,

Fig. 3. Partition-column chromatography of oestradiol-17 β , 16-epioestrinol and oestrinol. Inert phase, Celite; stationary phase, methanol-water (70:30, v/v); mobile phase no. 1, benzene-hexane (55:45, v/v); mobile phase no. 2 ($\uparrow$), benzene.

0.37 cm.²; stationary-phase cross-sectional area, 0.36 cm.²; retention volume, 4.3 ml.

Elution pattern of ketonic oestrogens

Fig. 4 shows the separation of 2-methoxyoestrone, oestrone and 16 α -hydroxyoestrone. In this experiment, 28 ml. of eluate between the 2-methoxyoestrone and oestrone fractions, and 48 ml. of eluate between the oestrone and 16 α -hydroxyoestrone fractions, were free of oestrogen. Under similar experimental conditions 16-oxo-oestrone was eluted with oestrone and 16-oxo-oestradiol-17 β with 16 α -hydroxyoestrone. This system showed excellent reproducibility as determined by the measurement of column parameters (as described above, Sudan Red was used). Typical column parameters were as follows: column

Fig. 4. Partition-column chromatography of 2-methoxyoestrone, oestrone and 16 α -hydroxyoestrone. Inert phase, Celite; stationary phase, methanol-water (70:30, v/v); mobile phase no. 1, hexane-benzene (98:2, v/v); mobile phase no. 2 ($\uparrow$), hexane-benzene (50:50, v/v).

Table 2. Recovery of oestrogens added to Celite and chromatographed on columns

Oestrogen added	Amount added (μg .)	No. of expts.	Percentage recovery (mean)
2-Methoxyoestrone	51	1	99
	20	1	90
Oestrone	67	2	94
	51	1	100
	20	1	97
	10	2	95
16 α -Hydroxyoestrone	37	1	85
	15	1	89
16-Oxo-oestradiol-17 β	16	1	94
Oestradiol-17 β	24	2	96
	12	3	92
	10	3	95
16-epioestrinol	47	1	91
	9	2	94
Oestrinol	25	1	93
	20	1	87

length, 11.6 cm.; column area, 0.88 cm.²; inert-phase cross-sectional area, 0.15 cm.²; mobile-phase cross-sectional area, 0.35 cm.²; stationary-phase cross-sectional area, 0.38 cm.²; retention volume, 4.1 ml.

Recovery of oestrogens added to Celite and chromatographed on columns

Table 2 shows the satisfactory recoveries of pure oestrogens after they were applied to Celite, transferred to a partition-chromatography column and eluted.

DISCUSSION

The experiments on the Girard reaction indicate that under appropriate conditions the ketonic and the non-ketonic oestrogens may be separated quantitatively and without destruction. There has been disagreement about the quantitative value of this procedure in oestrogen methodology. Westerfeld, Thayer, MacCorquodale & Doisy (1938) reported that 70–90% of added oestrone may be recovered in the ketonic fraction after the reaction. Pincus & Pearlman (1942) used the reaction successfully in the separation of urinary oestrone and oestradiol-17 β . Levitz, Spitzer & Twombly (1956) carried out the original procedure of Girard & Sandulesco (1936) on 16-[16-¹⁴C]-oxo-oestradiol-17 β and found radioactivity in the non-ketonic fraction. Marrian (1957) discovered that 16-oxo-oestradiol-17 β and 16 α -hydroxyoestrone are both unstable in the Girard reaction in hot solution, but stable when it is performed at room temperature. The latter finding has been confirmed by the present investigation. In addition, we have found that a small amount of the 16 α -hydroxyoestrone was in the non-ketonic fraction, although the Girard reaction was carried out at room temperature. Dr W. Klyne (personal communication) has suggested that the 16 α -hydroxyl group may hinder the formation of the Girard complex at the 17-position. Other possible explanations include the formation of a water-insoluble azine (Bates, 1952) or the hydrolysis of the Girard complex during neutralization with alkali. In addition, our studies demonstrate that the Girard reaction can be used to separate 2-methoxyoestrone and oestrone satisfactorily from the non-ketonic oestrogens.

The partition columns and method of residue transfer described in this report differ from those of Bauld (1955); they have three distinct advantages. First, it is now possible to separate and measure six instead of three oestrogens. Secondly, the technique of residue transfer avoids the difficulty of transferring polar steroids in non-polar solvents. Thirdly, the high percolation rate is a valuable time-saver.

Recently, this method as developed for pure

steroids has been incorporated into procedures for the separation and measurement of urinary oestrogens (Givner, Bauld & Vagi, 1960) and tissue oestrogens (Miss J. Mitchell and Dr R. Hobkirk, personal communication).

SUMMARY

1. A new chemical method which separates and quantitatively measures pure 2-methoxyoestrone, oestrone, 16 α -hydroxyoestrone (or 16-oxo-oestradiol-17 β), oestradiol-17 β , 16-*epio*oestrinol and oestrinol is described.

2. Experimental conditions for the Girard reaction resulting in quantitative recoveries of these oestrogens have been developed.

3. A technique for the quantitative transfer of absorbed oestrogen on Celite to a partition chromatogram is described.

4. Partition columns for the separation of 2-methoxyoestrone, oestrone, 16 α -hydroxyoestrone (or 16-oxo-oestradiol-17 β) and for the separation of oestradiol-17 β , 16-*epio*oestrinol and oestrinol are described.

5. The possible applications of this method are discussed.

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A Chemical Method for the Quantitative Determination of 2-Methoxy-oestrone, Oestrone, Ring D α -Ketolic Oestrogens, Oestradiol-17 β , 16-*epi*Oestriol and Oestriol in Human Urine

By M. L. GIVNER,* W. S. BAULD† AND KITTY VAGI

Department of Metabolism and McGill University Clinic, Montreal General Hospital, Montreal, Canada

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The object of this investigation was to develop a method for the quantitative determination of 2-methoxyoestrone, oestrone, ring D α -ketolic oestrogens, oestradiol-17 β , 16-*epi*oestriol and oestriol in human urine. The development of the method began with a detailed study of the experimental conditions required for quantitative separation of pure ketonic and non-ketonic oestrogens by means of the Girard reaction and the development of partition columns for the separation of the oestrogens in each of the Girard fractions (Givner, 1959; Givner, Bauld & Vagi, 1960).

The technique described in this report involves enzymic hydrolysis of the urine, ether extraction, separation of the ketonic from the non-ketonic oestrogens by the Girard reaction, the separation of the individual oestrogens by column-partition chromatography on Celite and colorimetric estimation. It differs from the methods of Brown (1955) and of Bauld (1956), which measure three urinary oestrogens, and from the method of Stimmel (1958), which separates five oestrogens by counter-current distribution.

Extensive specificity studies on the urinary-oestrogen fractions are also described. In addition, the method is evaluated for accuracy, adaptability and sources of error.

* Fellow of the Salmon Foundation for Medical Research. Present address: Department of Endocrinology, Southwest Foundation for Research and Education, San Antonio, Texas, U.S.A.

† Deceased.

METHODS

Purification of materials. Diethyl ether, benzene, hexane, ethanol, methanol, Kober reagents, Girard reagent P, Celite and asbestos were purified as described by Givner *et al.* (1960). Glusulase, a *Helix pomatia* extract (Endo Laboratories, New York) and bacterial β -glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used as received. Blue tetrazolium reagent (dianisole-bis-4:4'-(3:5-diphenyl)tetrazolium chloride was recrystallized from ethanol (96 %, by wt.) by the addition of diethyl ether. pH papers (ranges pH 2-10 and pH 9-11) were purchased from Fisher Scientific Co., Montreal. Glassware was cleaned as previously described (Bauld, 1956).

Collection of urine. Urine samples were collected for 24 hr. without preservative and stored at 5° if necessary.

Fractionation of urine

The purification procedure for urine is described below; letter references are to the flow sheet of Fig. 1.

Hydrolysis of late-pregnancy urine (A). A 50 ml. (approx.) portion of a 24 hr. urine sample was adjusted to pH 5.2 with 0.1 N-acetic acid or 0.1 N-NaOH and then hydrolysed with Glusulase (500 Fishman units of β -glucuronidase/ml. of urine) for 24 hr. at 39 $\pm$ 0.5°. The units were as defined by the supplier and were based on the assay procedure of Talalay, Fishman & Huggins (1946). The urine was cooled to 5° and two 20 ml. portions of the hydrolysed urine were transferred to separating funnels, each containing 80 ml. of water.

Hydrolysis of male urine (A). A 24 hr. urine sample was diluted to 2.5 l. with water; a 250 ml. portion (approx.) was adjusted to pH 6.5, and hydrolysed with bacterial β -glucuronidase (Sigma Chemical Co.), 20 units/ml. of urine for 48 hr. at 37 $\pm$ 0.5°, and then cooled to 5°. The