The lower inhibitory activity of TEM at pH 8.3 was not due to its decomposition in the alkaline solution (Table 1, TEM*). Fig. 1 shows that, although the activity of the HN 2 steadily increased with increasing pH, TEM appeared to exert its optimum inhibitory activity at about pH 6.3. The data in Table 2 show that the ethyleneimines are irreversible inhibitors of AChE. That this was not specifically due to the use of stromata at pH 6.3 is shown by the reversibility of the inhibition induced by eserine and neostigmine at that pH.

Fig. 2 shows that TEM brings about a progressive inhibition of AChE. The inhibition is irreversible and the lowest concentration of TEM which would cause 50% inhibition in an unlimited time was 3.0×10^{-4} M. A limit was placed on the reduction in concentration, since TEM decomposes slowly in aqueous solution and AChE is not so stable at pH 6.3 as at pH 7.4 or 8.3. A very small volume of TEM solution is as effective as a large volume in bringing about inhibition of the AChE of stromata, indicating a small uptake of TEM by the stromata. The rate of decomposition of TEM in solution was not appreciably accelerated in the presence of stromata. No difference was found in the action of TEM on ox or human stromata, and it was immaterial whether the stromata were fresh, preserved with chloroform water or previously freeze-dried.

SUMMARY

1. A convenient method of examining drugs for antiacetylcholinesterase activity at pH 6.3 by the

use of insoluble erythrocyte envelopes has been developed.

2. The inactivity of the epoxides and methane-sulphonyloxy derivatives as anticholinesterases has been confirmed.

3. The ethyleneimines and an HN 2 derivative show greatly increased anticholinesterase activity at pH 6.3 compared with pH 7.4 or 8.3.

4. Anticholinesterase activity of the ethyleneimines is progressive and irreversible.

5. Only very small quantities of TEM are taken up by erythrocyte stromata.

REFERENCES

- Aldridge, W. N. & Davison, A. N. (1952). *Biochem. J.* **51**, 62.
- Alexander, P., Fox, M., Stacey, K. A. & Smith, L. F. (1952). *Biochem. J.* **52**, 177.
- Augustinson, K. B. (1948). *Acta physiol. scand.* **15**, Suppl. 52, 43.
- Bergel, F. (1955). *J. Pharm., Lond.*, **7**, 297.
- Bergmann, F. & Shimon, A. (1953). *Biochem. J.* **55**, 50.
- Bullock, K. (1955). *Chem. & Ind.* p. 36.
- Davison, A. N. (1953). *Biochem. J.* **54**, 583.
- Gage, J. C. (1953). *Biochem. J.* **54**, 426.
- Ross, W. C. J. (1953). *Advanc. Cancer Res.* vol. 1, p. 397. New York: Academic Press Inc.
- Voegtlin, C., Fitch, R. H., Kuhler, H., Johnson, J. M. & Thompson, J. W. (1955). *Nat. Inst. Hlth Bull.* no. 164, 1.
- Watkins, W. M. & Wormall, A. (1952). *Biochem. J.* **52**, 365.
- Wilson, I. B. & Bergmann, F. (1950). *J. biol. Chem.* **185**, 479.

A Method for the Determination of Oestriol, Oestrone and Oestradiol-17 β in Human Urine by Partition Chromatography and Colorimetric Estimation

By W. S. BAULD

Department of Metabolism and McGill University Clinic, The Montreal General Hospital,
and Biochemistry Department, University of Edinburgh*

(Received 10 October 1955)

The purpose of this investigation was to devise a method for the determination in human urine of oestriol, oestrone and oestradiol-17 β , suitable for routine use and of sufficient sensitivity for the determination of the oestrogens excreted during the menstrual cycle. This investigation has been conducted independently of that reported by Brown (1955*a, b*), with whom close liaison was maintained at

all stages. The development of the method began with a review of methods of hydrolysis of urinary oestrogens (Marrian & Bauld, 1951). This was followed by investigation of the colorimetric determination, the column partition chromatography, and the sources of error in extraction of urinary oestrogens (Bauld, 1954, 1955*a, b*).

The final method of determination, described in this report, involves acid hydrolysis of the urine, separation of the ether extract into oestriol and

* Present address.

oestrone-oestradiol fractions, followed by column partition chromatography and colorimetric determination. It differs from the method of Brown (1955*b*), which uses methylation and adsorption chromatography for purification and separation. Results obtained in parallel assays by the two methods of the urine of men and of pre- and post-menopausal women are in good agreement (see Marrian, 1955).

With the present procedure four urine specimens can be analysed in 9–10 man-hours. The results obtained in analyses of urine excreted during the menstrual cycle are reproducible, and the method appears to be specific for oestrogens. With urines containing very small amounts of oestrogens the corrected optical densities in the colorimetric measurements are very small and the percentage error correspondingly large, by reason of instrumental limitations. The method is satisfactory for quantities of the order of 5–10 $\mu\text{g./day}$; Brown (1955*c*) has shown that such a method is adequate for demonstrating the variation of urinary oestrogens during the menstrual cycle.

METHODS

Purification of materials

Reagents used are A.R., unless otherwise stated. Solvents are distilled in all-glass apparatus.

Benzene. This reagent (Mallinckrodt, thiophen-free) is distilled from liquid paraffin (B.P., 50 ml./l.), and redistilled.

Ethanol. 95% ethanol (unmatured spirits) is allowed to stand over *m*-phenylenediamine with occasional shaking for at least 1 week, then distilled twice.

Ether. This is shaken with 0.8*M*- FeSO_4 in 0.4*N*- H_2SO_4 (3 $\times$ 100 ml./l.), washed with water (2 $\times$ 50 ml./l.) and distilled.

Ethylene dichloride (I.C.I., metal or stoneware containers, or Anachemia, Technical Grade). The solvent is poured through a column of silica gel (1 in. in diameter, 12 in. of length being used for each litre) at a rate of 4–6 ml./min. and distilled within 24 hr. of use.

Methanol. The solvent (Anachemia, acetone-free) is distilled from liquid paraffin (50 ml./l.).

Celite 535 (Johns Manville and Co. Ltd.). This is heated at 400° for 4 hr., stirred with an excess of conc. HCl (11*N*) and allowed to stand overnight. The solid is washed with water until the washings are free of Cl^- (AgNO_3 test), Fe^{3+} (SCN^- test), and neutral. The material is then dried at 110° for 48 hr., cooled in a vacuum desiccator and stored in containers with tightly fitting glass stoppers.

Cleaning of glassware

Glassware is rinsed after use with tap and distilled water. When visibly dirty it is allowed to stand in a chromic acid-sulphuric acid mixture for 12–24 hr. It is then rinsed with water, soaked in ethanol to destroy traces of chromic acid, washed with a vigorous stream of water for 2–3 min. and finally rinsed in distilled water.

Preparation of reagents

Concentrated carbonate solution, pH 10.5 (approx.), is prepared by diluting 130 ml. of 5*N*-NaOH to 1 l. with *m*- NaHCO_3 .

Reagent for oestradiol-17 β . NaNO_3 (10 mg.) and quinone (certified chemical, 20 mg.) in 1 l. of H_2SO_4 (60%, v/v) are warmed (at approx. 50°) until the solution turns light green and opalescent. Quinol (20 g. of B.D.H. Laboratory Reagent) is added, dissolved by further warming and shaking, and the solution (after cooling) is filtered through sintered glass (porosity no. 4, fine).

Reagents for oestrone and oestriol. These reagents are prepared as above, but with 66% (v/v) and 76% (v/v) H_2SO_4 respectively as solvents.

Collection of urine

A complete 24 hr. urine specimen is collected without preservative, stored at 4° if necessary, and analyses are begun within 48 hr.

Fractionation of urine

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

Hydrolysis (A). The 24 hr. urine specimen is diluted to 2.5 l. with distilled water and mixed thoroughly, and two 500 ml. portions are placed in 1 l. round-bottomed flasks. The samples are brought to the boiling point under reflux condensers, 75 ml. of conc. HCl is added and boiling continued for 1 hr.

Separation of the acid fraction. The cooled solutions are extracted with ether (1 $\times$ 150 ml., 3 $\times$ 125 ml.), interfacial solid residues being discarded after vigorous shaking of the combined extracts. The ether solutions are submitted to the following procedures: (B) Washed with 100 ml. of conc. carbonate solution (pH 10.5 approx.), which is then discarded. (C) Shaken with 25 ml. of 2*N*-NaOH. (D) The aqueous phase, without removal from the separating funnel, is partially neutralized with 100 ml. of *m*- NaHCO_3 ; the ether and aqueous phases are again shaken and the aqueous phase is discarded. (E) Washed with *m*- NaHCO_3 (1 $\times$ 25 ml.), and water (1 $\times$ 12.5 ml.); both aqueous phases are discarded. The ether solutions are then distilled to dryness. When the 24 hr. urine volume is 1250 ml. or less, one-half of the quantities given above are used.

Benzene-water partition (F). The residues are dissolved in 1.5 ml. of ethanol and transferred to separating funnels with 25 ml. of benzene. The solutions are washed with water (2 $\times$ 25 ml., 2 $\times$ 12.5 ml.), giving an aqueous phase which contains oestriol. The benzene is removed *in vacuo*, giving the oestrone-oestradiol-neutral fraction.

Preliminary purification of the aqueous phase, containing oestriol (G–K). The aqueous washings are treated with 7.5 ml. of 10*N*-NaOH (G), boiled for 30 min. under reflux (H), and extracted with 100 ml. of ether (I), this being discarded. The aqueous phase is then brought to pH 9.3–9.5 (J) by passing CO_2 from a manifold into separating funnels containing the extracts. One limb of the manifold is led into a similar funnel containing 75 ml. of water and 7.5 ml. of 10*N*-NaOH and thymolphthalein; this separate funnel serves as an indicator for the partial neutralization of the funnels containing urine extracts. With a manifold diameter of 1 in., and capillary-glass tubing of 1 mm. bore, the rate of gas flow into the funnels is sufficiently uniform that no

further check on the pH is required. These solutions are then (K) extracted with ether (4×40 ml.), and the combined extracts washed with $m\text{-NaHCO}_3$ (1×5 ml.) and water (2×5 ml.). The ether is then distilled, leaving the impure oestriol fraction.

Chromatography of the impure oestriol fraction (L). Chromatograms are prepared as previously described (Bauld, 1955a), with the following characteristics: mobile phase, ethylenedichloride; stationary phase, 70% methanol; columns 1 cm. $\times$ 10 cm. are packed from a slurry containing 1 ml. of stationary phase per g. of Celite; diameter of holes in packing plunger, 0.7 mm.; temp. $18 \pm 0.5^\circ$; percolation rate of solvent, 10–12 ml./hr. The residues are dissolved in 1 ml. of mobile phase, transferred by pipettes carefully to the tops of the chromatograms and allowed to pass through the column until the top is just dry. The transfer procedure is repeated twice with 1 ml. washings of mobile phase. When the second wash enters the chromatogram, collection of eluate is begun and a reservoir containing mobile phase is

fitted to the top of the column. The eluate from 0 to 14 ml. is discarded and the next 16 ml. collected giving the oestriol fraction. The solvent is removed *in vacuo*, the residue dissolved in 3 ml. of ethanol and a 2 ml. portion transferred to a colour reaction tube, quinol (50 ± 5 mg.) added, and the solvent removed by heating in a boiling-water bath with a stream of filtered air directed on the surface of the solution.

Chromatography of the oestrone-oestradiol-neutral fraction (M). Chromatograms are prepared as previously described (Bauld, 1955a), with the following characteristics: mobile phase, benzene; stationary phase, 0.8N-NaOH; columns 1 cm. $\times$ 12 cm. are packed from a slurry containing 0.8 ml. of stationary phase/g. of Celite; diameter of holes in packing plunger, 1.0 mm.; temp. $18 \pm 0.5^\circ$; percolation rate of solvent, 10–12 ml./hr. The residues are applied to the chromatograms as described above for chromatography of the impure oestriol fraction. The eluate from 0 to 10 ml. (neutral fraction) is discarded, the end of the column rinsed with a stream of benzene, and the eluate from 10 to 30 ml.

Fig. 1. Flow sheet of the procedure for separation of oestriol, oestrone and oestradiol-17 β from urine.

collected, giving the impure oestrone fraction. The next 10 ml. is discarded and the mobile phase changed to ethylene dichloride-benzene (3:1, v/v) and 50 ml. collected, giving the impure oestradiol fraction.

Slight variations between different workers in the technique of packing are inevitable (Bauld, 1955*a*). For this reason the exact elution pattern of the oestrogens should be determined in any laboratory using this method and fractions taken accordingly.

Final purification of the impure oestrone fraction (N). This fraction is evaporated to dryness *in vacuo*, boiled under reflux for 30 min. with 10 ml. of *N*-NaOH, acidified with 1 ml. of 12*N*-H₂SO₄ and extracted with benzene (1 × 20 ml.). The extracts are washed with 0.5*M*-Na₂CO₃ (1 × 4 ml.) and water (2 × 4 ml.) and transferred to colour-reaction tubes. Quinol (50 ± 5 mg.) is added and the solvent removed by heating at 80–90° in a stream of filtered air. This gives the oestrone fraction.

Final purification of the impure oestradiol fraction (O). This is treated exactly as described above for the impure oestrone fraction and yields the oestradiol fraction.

Colour development on purified extracts

The appropriate reagent (2.6 ml. for oestradiol-17β and oestriol; 3.0 ml. for oestrone) is added to the oestrogen and quinol residues contained in test tubes (23 mm. × 150 mm.). These are heated for 20 min., shaken twice to ensure adequate mixing and solution of the quinol. The solutions are then cooled in a bath of cold water (approx. 15°), 50 ± 5 mg. of quinol is added, and dilution carried out as follows: oestradiol-17β, with 0.7 ml. of reagent; oestriol, with 0.7 ml. of water; oestrone, with 0.3 ml. of water. The tubes are then shaken 5–10 times and reheated for 15 min. with two shakings during this time to dissolve the quinol. The cooled solutions are transferred to 1 cm. cuvettes and their optical densities measured at 480, 512.5 and 545 mμ. for oestrone and oestriol and at 480, 515 and 550 mμ. for oestradiol-17β. These measurements can be done on the Unicam SP. 600 or the Beckman DU spectrophotometer; if a Beckman Model B spectrophotometer is used, the line voltage must be stabilized. Optical-density readings (*D*) are corrected by applying the following equations (Allen, 1950):

oestriol and oestrone corrected readings

$$= D_{512.5 \text{ m}\mu} - \frac{1}{2}(D_{480 \text{ m}\mu} + D_{545 \text{ m}\mu});$$

oestradiol corrected reading

$$= D_{515 \text{ m}\mu} - \frac{1}{2}(D_{480 \text{ m}\mu} + D_{550 \text{ m}\mu}).$$

Precautions taken in analyses

In this method oestrogens are being determined at a concentration of approximately 5 μg./l. of the original urine. Meticulous care must therefore be taken to avoid contamination of glassware, mouths of reagent bottles and separating funnels, ground-glass joints, stoppers, air jets, etc. Experience has shown that three types of contamination are important and examples of these are described below.

Heavy-metal contamination. Oestrogens in small amounts undergo oxidation in the presence of heavy metals. These reactions are of quantitative significance when microgram quantities of oestrogen are involved. Glassware is therefore kept free of dust and traces of cleaning solution. The flasks used for NaOH treatment become pitted in time and tend to retain traces of chromic acid. They are treated with chromic acid-sulphuric acid only when necessary, and on these occasions are soaked overnight in ethanol before thorough rinsing.

Contaminants increasing background colour in Kober reaction. Quinol is used to protect the oestrogens from oxidation during the removal of solvents from the colour-reaction tubes. Care is taken to ensure that no traces of carbonate on the lips of separating funnels used for extraction of oestrone and oestradiol-17β after NaOH treatment are carried into the Kober tubes.

Contamination from solvents. This is an important source of error, and simple tests of solvent purity are applied. Benzene and ethylene dichloride, after purification, must give no colour on being shaken with H₂SO₄. The residue from distillation of 50 ml. of these solvents should give no visible colour with 1 ml. of H₂SO₄. Purified methanol and ethanol (0.2 ml.) should not show a perceptible colour when heated with oestriol reagent (3 ml.) for 5 min. in a boiling-water bath.

In the later stages of the method, small volumes of solvents are used for extraction and washing. Careful attention is paid to quantitative transfer and the avoidance of loss. Special care is required in rinsing the glass bead present during evaporation of small volumes of solvent, since it frequently has most of the residue adsorbed on its surface.

RESULTS

Elimination from urine extracts of substances yielding a brown colour in the modified Kober reaction

Examples are given in Table 1 of the degree of purification (expressed as percentage decrease in

Table 1. Effectiveness of various stages in purification

Two portions (0.1 vol.) of 24 hr. male urine specimens were purified by the procedures below; the individual stages used are indicated by letter references to Fig. 1 and are shown as inclusive. The appropriate colour reaction was carried out on the final extracts and the purification obtained in an individual stage calculated from the uncorrected optical densities (o.d.) at 512.5 mμ. as follows:

$$\text{Purification (\%)} = \frac{\text{o.d. (procedure 1)} - \text{o.d. (procedure 2)}}{\text{o.d. (procedure 1)}} \times 100. \text{ See also text.}$$

Expt.	Stage examined	No. of urines	Procedure 1	Procedure 2	Purification	
					Mean	Range
1	<i>J</i>	4	<i>A, E, F, K</i>	<i>A, E, F, J, K</i>	40	33–43
2	<i>G</i>	4	<i>A, E, F, J, K</i>	<i>A, E, F, G, J, K</i>	41	28–57
3	<i>I</i>	6	<i>A–G, J, K</i>	<i>A–G, I, J, K</i>	40	14–70
4	<i>H</i>	7	<i>A–G, I, J, K</i>	<i>A–K</i>	60	37–78
5	<i>L</i>	4	<i>A–K</i>	<i>A–L</i>	63	50–71
6	<i>N</i>	11	<i>A–M</i>	<i>A–N</i>	39	20–66
7	<i>O</i>	5	<i>A–M</i>	<i>A–O</i>	50	38–67

Table 2. *Recovery of oestrogens added to water at different stages of the fractionation procedure*

Oestrogens in the amount shown below were added at various stages of the extraction procedure to water of the volume shown for the aqueous phase under Methods. The purification stages used are indicated by letter references to Fig. 1. See also text.

Expt.	Oestrogen added	Amount added	No. of expt.	Stages investigated	Recovery (%)		
					Mean	S.E.	Range
1	Oestriol	10, 25	10	A-E	90	0.6	88-94
2	Oestriol	25	8	F	100	1.0	97-103
3	Oestriol	10, 25	8	F-K	99	0.7	96-103
4	Oestriol	150	12	L	95	0.7	91-97
5	Oestriol	25, 150	6	A-L	89	1.3	84-92
6	Oestrone	25, 50	12	A-F	98	0.5	95-100
7	Oestrone	150	12	M	95	0.9	91-100
8	Oestrone	10	12	N	94	0.3	92-96
9	Oestradiol	25, 50	9	A-F	100	0.7	97-104
10	Oestradiol	150	12	M	97	1.1	91-101
11	Oestradiol	10	12	O	95	0.6	92-98

gross optical density at 512 m μ .) obtained in different steps of the fractionation procedure. Expt. 1 demonstrates the purification obtained when the oestriol fraction was re-extracted from a slightly alkaline medium (pH 9.3) rather than from water, and Expt. 2 shows the increased purification resulting when this was combined with prior strong alkalization. Ether extraction of the strongly alkaline solution before the partial neutralization (Expt. 3) removed an additional amount of impurity; this contained 3-4 % nitrogen. Refluxing in N-NaOH increased the amount of extraneous material left in the aqueous phase; this was visibly apparent and is shown quantitatively in Expt. 4. Purification obtained in partition chromatography of the impure oestriol fraction is shown in Expt. 5. The removal of impurities by saponification of the impure oestrone and the impure oestradiol fractions is shown in Expts. 6 and 7.

Omission of steps B-D (Fig. 1) from the complete procedure did not affect appreciably the purity of the final fractions. Their inclusion, however, minimized emulsions in the benzene-water partition (stage F, Fig. 1) and markedly decreased the brown pigments formed on top of the oestrone-oestradiol-neutral chromatograms.

Steam distillation of the hydrolysed urine, or of the butanol extraction before hydrolysis, gave only very slight and variable purification of the final product. In general, hydrolysis with 12N sulphuric acid gave less charring and more highly coloured ether extracts, but there was little difference in the purity of the final products.

Recovery of oestrogens added to water

The various stages in purification were included in the method only when it could be demonstrated that, in addition to their efficacy in purification, they caused little or no loss of oestrogen. Stage-by-stage investigation of the recovery of oestrogens from pure solution was carried out by adding known

Table 3. *Recovery of oestrogens added to normal male urine after hydrolysis*

Oestrogens were added to acid-hydrolysed normal male urine (0.2 vol. of the 24 hr. specimen) and the total oestrogen content of the urine was determined by the complete procedure described under Methods. Simultaneous analyses (in duplicate) were carried out on the urine to determine its endogenous oestrogen content, this value being used to correct the recovery. The amounts of oestrogen added are expressed in terms of the 24 hr. specimen. Twelve experiments were carried out with each compound at each concentration. See also text.

Oestrogen added	Amount added (μ g.)	Recovery (%)	
		Mean $\pm$ S.E.	Range
Oestriol	25	90 $\pm$ 1.7	82-99
Oestriol	10	87 $\pm$ 2.1	77-97
Oestriol	5	81 $\pm$ 3.1	60-97
Oestrone	25	90 $\pm$ 1.5	81-100
Oestrone	10	91 $\pm$ 1.2	85-97
Oestrone	5	90 $\pm$ 2.0	60-103
Oestradiol	25	88 $\pm$ 1.6	80-96
Oestradiol	10*	70 $\pm$ 1.8	60-78
Oestradiol	5*	74 $\pm$ 7.1	38-100
Oestradiol	5	84 $\pm$ 2.0	72-94

* Step O, Fig. 1 was omitted.

amounts of oestrogens to a volume of water equal to that of the aqueous phase in the particular procedure being examined and described under Methods. The results of these investigations are shown in Table 2.

Recovery of oestrogens added to hydrolysed urine

Table 3 gives the results of recovery experiments when oestrogens were added to acid-hydrolysed urine (0.2 vol. of a 24 hr. normal male specimen). Two similar portions of the same urine were also analysed in order to correct the recovery figures for the endogenous-oestrogen content.

Results obtained with higher levels of oestrogen and an earlier version of the method have previously been described (Bauld, 1955*b*). In this earlier procedure, the oestriol fraction was extracted with ether before as well as after refluxing with alkali, and a mild reducing agent was added during this stage. That these steps were unnecessary was shown in a series of ten experiments in which oestriol (1 $\mu\text{g.}$) was added to the urinary extract at step *G* in the present procedure (see Fig. 1) and was recovered to the extent of 92%, s.e. 1.6. Moreover, in the earlier experiments, the oestrone fraction was not saponified after hydrolysis. In ten experiments with different urines, the recovery of oestrone (1 $\mu\text{g.}$) from step *N* was 92%, s.e. 0.9, indicating negligible loss in saponification.

Assessment of the method

Specificity. This is determined by the absence of non-oestrogen chromogenic material with a peak of absorption between 480 and 550 $m\mu$. Fig. 2 shows for a typical urine the wavelength/absorption curves for (*A*) the oestriol fraction (measured), (*B*) the amount of pure oestriol calculated to be present in this fraction by the colour-correction formula of Allen (1950) (measured), and (*C*) the non-oestrogen chromogenic material of the oestriol fraction (obtained by subtracting *B* from *A*). The curve obtained for the reagent blank of the fractionation

procedure (measured) is shown in (*D*). Similar results have been obtained for oestrone and oestradiol fractions. This specificity was proven for all three fractions in a wide variety of urines from normal and pathological cases. Large quantities of phenolphthalein in the urine were not completely removed by the purification procedures and caused a non-linear response from 460 to 480 $m\mu$, so that urines from patients receiving this drug cannot be analysed by this method.

Reproducibility. The agreement between duplicates obtained by one worker in a series of consecutive analyses carried out on male and female urines is shown in Table 4.

Sample analyses. The results of typical duplicate analyses (including spectrophotometer readings) of a normal female urine (9th day of cycle) and a menopausal female urine are shown in Table 5.

Table 4. Variations between the results of duplicate analyses

For details, see text.

Oestrogen ($\mu\text{g.}/24 \text{ hr.}$)	No. of Expt.	Variation (%)		
		Mean	s.e.	Range
0-4	18	15	2.8	0-38
4-16	36	4	0.9	0-19
16-25	12	5	0.7	0-10

Fig. 2. Wavelength/absorption curves of Kober colours produced by (*A*) the oestriol fraction from one-fifth of a 24 hr. urine excreted in the luteal phase of a normal cycle, calculated by the correction formula to contain 2.1 $\mu\text{g.}$ of oestriol; (*B*) 2.1 $\mu\text{g.}$ of oestriol; (*C*) the non-oestrogen contaminants of this oestriol fraction, calculated by subtracting (*B*) from (*A*); (*D*) the oestriol fraction reagent blank.

Table 5. *Sample analyses*

	Optical densities measured			Calc.		
	480 m μ .	512.5 or 515 m μ .	545 or 550 m μ .	Corrected O.D.	Oestrogen	
					μ g.	μ g./24 hr.
Case 1. 35-year-old female, ninth day of cycle						
Oestriol	0.480	0.490	0.311	0.094	5.3	26.5
	0.472	0.484	0.299	0.098	5.5	27.5
Oestrone	0.432	0.411	0.300	0.045	1.4	7.0
	0.502	0.479	0.358	0.049	1.5	7.5
Oestradiol	0.382	0.340	0.252	0.023	0.8	4.0
	0.350	0.322	0.252	0.021	0.7	3.5
Case 2. 32-year-old female, early menopause						
Oestriol	0.135	0.115	0.070	0.012	0.7	3.5
	0.152	0.130	0.085	0.011	0.6	3.0
Oestrone	0.290	0.240	0.170	0.010	0.3	1.5
	0.280	0.240	0.180	0.010	0.3	1.5
Oestradiol	0.147	0.123	0.090	0.005	0.2	1.0
	0.115	0.100	0.070	0.007	0.2	1.0

DISCUSSION

The hydrolysis of conjugated urinary oestrogens, the practicability of partition chromatography for fractionation of the free oestrogens, and the development of the basic extraction and purification procedure from the earlier studies of Clayton (1949) have already been described (Marrian & Bauld, 1951; Bauld, 1955*a, b*). The method reported in the present communication also includes stages designed to remove two principal types of non-oestrogen chromogenic material: brown impurities containing nitrogen, and substances which on oxidation form brown products.

The first of these two types of contaminants is neutral or slightly basic in reaction. It was divided into hydrophilic and lipophilic components in the benzene-water partition (step *F*; this and subsequent letter references are to Fig. 1). The lipophilic fraction was eluted with the neutral fraction from the chromatogram (*M*) before oestrone. The hydrophilic neutral and basic fraction was extracted with ether at step *I*. In urines of high indican content, this residue contained pink and violet pigments which behaved on alumina chromatography in the same way as did indigo red and indigo blue (cf. Rimington, 1946). Presumably this explains, at least in part, the nitrogen found in this fraction.

The second principal type of impurity consists of substances very susceptible to oxidation to brown products. Boscott (1949) used mild reducing agents during extraction, but reoxidation occurs so readily at subsequent stages (e.g. during chromatography or evaporation of solvents) that his technique has not been used in the present method. The major portion of this group of impurities was readily

removed, since on oxidation they were more easily extracted from ether into dilute alkali. Thus making the aqueous fraction strongly alkaline in step *G* (to effect oxidation) before re-extraction from pH 9.3 markedly increased the impurities left in the weak alkaline phase; this is shown in Expt. 2, Table 1. When Brown (1952) extended the principle to the initial ether extraction, steps *C* and *D* were included, since purification at an earlier stage minimized emulsification during the benzene-water partition (*F*) and decreased the residue applied to the partition chromatogram (*M*). Part of this type of impurity, however, was extractable by ether from dilute alkali and persisted into the final colour reaction. It was thought these residual contaminants might be esters of phenolic acids. Since oestrogens are stable in boiling dilute sodium hydroxide (Diczfalusy, 1953; see also Table 2, Expts. 3, 8 and 11), saponification was tried and considerable purification achieved as shown in Table 1, Expts. 4, 6 and 7. This step was used late in the procedure to decrease solvent blanks and to minimize the possibility that substances in the cruder extracts might facilitate oxidation of the oestrogens (cf. Bauld, 1955*b*).

The various purification stages were adopted in order to decrease the non-oestrogen chromogenic material and thus minimize the colour correction. In addition, however, two of the stages (*B, O*) were included because they increased the percentage recovery of added oestrogens. These have previously been discussed (Bauld, 1955*b*) and the improved recovery of oestradiol in certain urines resulting from the inclusion of step *O* is readily apparent from Table 3.

Even with the extensive purification procedure, an appreciable amount of non-oestrogen chromo-

genic material is still present in the final colour reaction. A colour correction is therefore necessary, and the method proposed by Allen (1950), based on the geometry of similar triangles, has been found suitable. This correction is valid only when the non-specific absorption does not show a peak in the range over which the optical-density measurements are taken. Fig. 2C shows that the non-specific absorption is linear from 480 to 550 m μ .

This linearity suggests a high degree of specificity for the present method. It is possible that purified urinary extracts may contain a non-oestrogen chromogen of $\lambda_{\max}$. 512 m μ . High specificity is, however, further supported by the close agreement, previously reported by Marrian (1955) between results obtained by this procedure and those obtained by the markedly different method of Brown (1955b). These comparisons were made when the impure oestrone fraction in the method reported in this paper was not being saponified. Under these conditions the brown-coloured impurities in the Kober reaction in Brown's procedure were 54% (45–68) less for oestrone, 49% (20–61) more for oestriol, and 41% (22–56) more for oestradiol, than in the present author's earlier procedure (Bauld, 1955b). In spite of these wide variations in the residual contaminants, the results of parallel assays were very similar (Marrian, 1955), indicating the reliability of the colour correction formula of Allen (1950).

The present investigation gave no indication of the accuracy of the complete procedure, since recovery experiments were carried out only with oestrogens added after hydrolysis. Preliminary investigations suggest a loss of 5–15% of oestradiol-17 β , and 0–10% of oestrone and oestriol in hydrolysis. The accuracy of the extraction procedure is apparent from Table 3. The scatter of the percentage recovery increased markedly at the lower levels. The loss of precision in urines of low oestrogen content is apparent in Table 4. With the low optical densities, the instrument error caused relatively wide variations in reading, and the sensitivity of the method is limited to the order of 5–10 $\mu\text{g./day}$.

Greater sensitivity would be obtained by the use of a fluorimetric method of estimation. In our hands, however, existing fluorimetric methods have been found to be very susceptible to instability or quenching by solvent or urinary residues. That this effect is common may be seen by the variety of correction formulae and quenching tests recently reported (Finkelstein, 1952; Veldhuis, 1953; Braunsberg, Stern & Swyer, 1955). It is therefore thought that at present the increased sensitivity of the fluorescence method is a poor substitute for the greater stability and specificity of the modified Kober reaction.

SUMMARY

1. A detailed procedure for the extraction, separation and purification of oestriol, oestrone and oestradiol-17 β in human urine is described.

2. The method involves: acid hydrolysis; ether extraction; separation of oestriol from oestrone and oestradiol-17 β by distribution between benzene and water; purification of oestriol by saponification and column partition chromatography; separation and purification of oestrone and oestradiol-17 β by column partition chromatography and saponification. The purified fractions are determined colorimetrically by an improved Kober reaction with a spectrophotometric correction for non-oestrogen chromogenic material.

3. The precautions necessary for satisfactory analyses and the efficiency of the various purification procedures are described.

4. The specificity, accuracy, precision and sensitivity of the method are discussed.

The author wishes to thank Professor G. F. Marrian, F.R.S., for his earlier supervision of, and continuing interest in this investigation; Dr J. B. Brown for invaluable discussions during the development of the method; and Mr J. Gordon and Miss M. Pehrson for skilled technical assistance. Grateful acknowledgement is made to the National Research Council of Canada, Charles E. Frost and Co., Montreal, and the Blanche Hutchinson fund of McGill University, for generous grants which enabled a laboratory for oestrogen assay to be equipped and operated in the Department of Metabolism of The Montreal General Hospital. The earlier part of this investigation was supported by grants to Professor Marrian from the Medical Research Council.

REFERENCES

- Allen, W. M. (1950). *J. clin. Endocrin.* **10**, 71.
- Bauld, W. S. (1954). *Biochem. J.* **56**, 426.
- Bauld, W. S. (1955a). *Biochem. J.* **59**, 294.
- Bauld, W. S. (1955b). *Mem. Soc. Endocrin.* **3**, 11.
- Boscott, R. J. (1949). *Abstr. 1st Int. Congr. Biochem., Cambridge*, p. 412.
- Braunsberg, H., Stern, M. I. & Swyer, G. I. M. (1955). *Mem. Soc. Endocrin.* **3**, 41.
- Brown, J. B. (1952). Ph.D. Thesis: University of Edinburgh.
- Brown, J. B. (1955a). *Mem. Soc. Endocrin.* **3**, 1.
- Brown, J. B. (1955b). *Biochem. J.* **60**, 185.
- Brown, J. B. (1955c). *Lancet*, **228**, 320.
- Clayton, B. E. (1949). Ph.D. Thesis: University of Edinburgh.
- Diczfalussy, E. (1953). *Acta endocr., Copenhagen*, **12**, suppl. 12.
- Finkelstein, M. (1952). *Acta endocr., Copenhagen*, **10**, 149.
- Marrian, G. F. (1955). *Mem. Soc. Endocrin.* **3**, 48.
- Marrian, G. F. & Bauld, W. S. (1951). *Acta endocr., Copenhagen*, **7**, 240.
- Rimington, C. (1946). *Biochem. J.* **40**, 669.
- Veldhuis, A. H. (1953). *J. biol. Chem.* **202**, 107.