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The Isolation and Estimation of the Steroid Oestrogens in Placental Tissue

By F. L. MITCHELL AND R. E. DAVIES

Jessop Hospital for Women, Sheffield, and Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, The University, Sheffield, 10

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Work on the biosynthesis of the natural oestrogens has been rendered very difficult in the past by the lack of quantitative methods of purification of the microgram quantities of these hormones which exist in tissue. Though there were several methods of estimating the pure oestrogens, they were not directly applicable to accurate estimations in urinary or tissue extracts because of the presence of impurities.

The rigorous purification of oestrone, oestradiol and oestriol has been the aim of several recent investigations (Engel, Slaunwhite, Carter & Nathanson, 1950; Boscott, 1951; Swyer & Braunsberg, 1951; Bauld, 1952) all of which deal with urinary extracts, whilst Diczfalusy (1953) and Ryan & Engel (1953*a, b*) have recently applied the counter-current distribution method of Engel *et al.* (1950) to tissue extracts. In this technique, however, non-specific fluorogenic material present, particularly in the early fractions, makes difficult the estimation of minute quantities of oestrogen by fluorimetry.

The methods to be described were developed in order to make possible further work on the *in vitro* synthesis of oestrogens in tissue slices. Initially this had been investigated by using a bio-assay. The normal method of injecting the oestrogen into spayed, adult female mice had not proved to be sufficiently sensitive, but the intravaginal insertion technique of Emmens (1941) produced 50% vaginal cornification with 1.0 μ mg. oestrone, and by this means it had been shown that full-term human placental tissue contained 30 Emmens mouse units/g. When this placental tissue was incubated in Warburg flasks for 3 hr., the oestrogenic potency

increased by approximately 100%. However, the large number of mice required for assay, and the lack of specificity, made it clear that chemical estimations were required.

After a study of existing chromatographic techniques, a method was developed which involved a preliminary chemical separation followed by paper partition chromatography and a special spotting technique, with elution of the spots and estimation by fluorimetry.

A preliminary account of part of this work has already been published (Mitchell, 1952).

EXPERIMENTAL AND RESULTS

Reagents

Ether (May and Baker Ltd., Dagenham), A.R., peroxide-free, was refractionated through a 100 cm. column packed with single-turn glass helices, and used the same day. Benzene, A.R., thiophen-free, was refluxed for 30 min. with conc. H_2SO_4 and refractionated through a similar 60 cm. column. This column was also used to refractionate the AnalaR carbon tetrachloride and methanol. Ethanol was B.P. quality (James Burrough Ltd., London). Diazotized *p*-nitrobenzeneazodimethoxyaniline (Fast Black Salt K) was from the Clayton Aniline Co. Ltd., Manchester. All other reagents were of AnalaR quality.

Extraction procedure

The procedure developed in the course of this work is described in Fig. 1. Fresh placenta was minced and mixed with an equal volume of 80% (v/v) ethanol within 30 min. of delivery. The method of extraction separated the oestrogens into 'free' (1), 'conjugated' (2), and 'protein-bound' (3) fractions (Diczfalusy, 1953). These were each further

Fig. 1. Extraction of 'free', 'conjugated' and 'protein-bound' oestrogens from tissue, and their purification and separation into oestriol, and oestrone:oestradiol fractions. All extracts were stored at -20° when not being actually handled. All processes were carried out at room temp. except where stated. Before distillation of solvents, extracts were first evacuated at room temp. to remove air, and the distillation subsequently carried out in a water-pump vacuum with a fine stream of nitrogen bubbles.

separated into an oestriol (4) and an oestrone plus oestradiol (5) fraction. These fractions (4 and 5) were each dissolved in 5 ml. of 90% (v/v) methanol and washed with 5 ml. of pentane; the pentane was back-extracted three times with 5 ml. 90% (v/v) methanol and the pooled methanol evaporated to dryness at 70° in a stream of N_2 . The residue was taken up in 0.2 ml. of ethanol for chromatography. The pentane wash (Ryan & Engel, 1953a) is not necessary for the 'protein-bound' fraction. The reason for its inclusion is to remove remaining greasy materials, which if present in large amounts (more than 5 mg.) in the final extract cause spreading of the spot on the chromatogram during equilibration.

The original ethanol extracts from 400 g. placental tissue contained approx. 16 g. of material, which was finally reduced to 2–10 mg. for chromatography.

Initial investigation of techniques for further purification and estimation

Coupling with azo dye. Heftmann (1950) separated pure oestrogens by coupling with a saturated aqueous solution of Fast Black Salt K to make azo compounds, followed by paper chromatography. Our sample of this salt solution had to be diluted 30 times with water to achieve results similar to those of Heftmann (1950).

In order to investigate the possibility of eluting the purple spots from the paper and estimating the oestrogens colorimetrically, an effort was first made to increase the R_F value of oestriol and decrease that of oestrone. These are respectively 0.07 and 0.95, and the spots tended in the one case to merge with the residue left on the origin, and in the other with an orange band which moved with the moving-phase front.

The oestrogens were first separated into an oestrone: oestradiol and an oestriol fraction by the preliminary extraction procedure. Heftmann's (1950) original solvent mixture was 200 ml. of toluene, 100 ml. of light petroleum (b.p. 35–60°), 30 ml. of ethanol and 70 ml. of water; after equilibration the top layer was used for the moving phase. This system was modified by increasing the amount of light petroleum to 250 ml. for the oestrone and oestradiol mixture, and decreasing it to 50 ml. for oestriol. The R_F values in the respective systems were then: oestrone, 0.91; oestradiol-17 β , 0.63; and oestriol, 0.30.

The parts of the paper containing the spots were cut up and placed in 5 × 0.5 in. test tubes, 1 ml. of benzene was added, and the tubes were gently shaken for a few min.; 0.5 ml. of the benzene was then removed and the colour density measured in the 0.5 ml. cells of the Spekker Photoelectric Absorptiometer using an Ilford Spectrum Blue-green 603 filter (transmission max. 490 m μ). The absorptions were measured against extractions from controls containing no oestrogen.

Fig. 2 shows that this method may be used for the separate estimation of oestrone and oestradiol, but for oestriol the slope of the calibration curve was very small, and efforts to make the reaction more sensitive failed. More accurate measurements could be made with a Beckman Spectrophotometer.

Attempts to couple the oestrogens present in the oestrone and oestradiol fraction of placental extract with Fast Black Salt K and to chromatograph the products gave long, dark-brown streaks on the paper. With the oestriol fraction, a spot was formed corresponding in every respect to that of pure oestriol, but the amount present could not be determined precisely by the above method because the slope of the calibration curve was so small (see Fig. 2).

Pure oestrone and oestradiol were added to the fraction which contained these oestrogens, and the mixture was coupled with Fast Black Salt K. The product still did not produce any spots on the chromatogram, and this shows that something present in the extract interfered with the method.

Countercurrent distribution. This method of Engel *et al.* (1950) for separating oestrogens in urinary extracts proved unsuitable for the analysis of normal placental extracts because of a large, but variable, non-specific background fluorescence. Diczfalussy (1953) has used this method, but points out that some overestimation occurs at low concentration of oestrogen, and that oestrone cannot be quantitatively separated from the non-specific fluorogenic contaminants.

Chromatography on alumina-impregnated paper. Using this technique (Bush, 1950), a method of ascending two-dimensional chromatography was evolved which would separate, on papers 6 in. square, part of the oestrogens reasonably free from impurities. The solvents were run to the edge of the paper and the separation achieved is shown in Fig. 3. The position of the oestrogens was found by the iodine method (Bush, 1950).

Many of the original disadvantages of this method have been removed (Bush, 1952), but small amounts of impurity still cause alteration of R_F values with streaking.

Chromatography by Celite:sodium hydroxide column. Apparently reliable results have been obtained by Swyer & Braunsberg (1951) when this method was applied to the very low amounts of oestrogens found in non-pregnancy urines. The method has been further perfected for urine by Bauld (1952, 1953), and Bitman & Sykes (1953) have investigated its use for separating pure oestrogens. When the technique was applied to tissue extracts, large amounts of non-specific, fluorogenic material still remained in the oestrogen-containing fractions.

Chromatography on paper impregnated with glycerol and propylene glycol and on a rubber column. Investigation of these techniques (Boscott, 1951; Nyc, Maron, Garst & Friedgood, 1951) showed that, though good separations were produced using pure oestrogens, tissue extracts gave considerable spreading and tailing of the spots in the paper method, and in both cases there was insufficient removal of impurities.

Fig. 2. Standard curves for oestrogens coupled with Fast Black Salt K after elution from chromatograms with benzene. ○—○, oestradiol; +—+, oestrone; ●—●, oestriol. Oestradiol and oestrone measured in 0.5 ml. cups of a Spekker Photoelectric Absorptiometer; oestriol in a Beckman Spectrophotometer. The absorptions were measured against extractions from blank runs containing no oestrogen.

Fig. 3. Two-dimensional chromatography of oestrogens on alumina paper (after Bush, 1950). For further details see text. The spots were detected by iodine and drawn in before they faded.

Reversed-phase partition chromatography on silicone-treated paper. This method (Kritchevsky & Tiselius, 1951), gave perfect results for pure steroids, but impurities caused streaking and drastic alteration of R_F values.

Reduction of extracts by lithium aluminium hydride. Some of the impurities in the 'phenolic' fractions may have been weak acids containing carboxyl groups. If these acidic groups could be reduced by means of lithium aluminium hydride, then these impurities could be easily removed.

Much impurity was removed from the extract by this means, but further purification was still necessary as shown by the amount of residue, and by the results of coupling with Fast Black Salt K. In the reduction process, oestrone was quantitatively reduced to oestradiol as judged by the intensity of the spot after coupling.

The use of Folin & Ciocalteu's reagent. If the oestrogens were heated with this phenol reagent (Folin & Ciocalteu, 1927), the colour changed from yellow to green, and became blue on addition of alkali (see Fig. 4).

For quantitative assay, the oestrogen was dissolved in ethanol, placed in 5 × 0.5 in. test tubes, the ethanol removed in a current of nitrogen and 0.4 ml. of the reagent (diluted with 2 vol. water) added. The tubes were then stoppered with cotton wool, heated in a boiling-water bath for 10 min., cooled and transferred to a water bath at 25°; 0.15 ml. of 25% (w/v) Na_2CO_3 solution was added, the stoppers were replaced, and the tubes left for 10 min. The colour intensity was then measured within 5 min. in the 0.5 ml. cells of the Spekker instrument, using an Ilford Spectrum Red 608 filter (transmission max. 680–700 $\text{m}\mu$.) (Fig. 4). Controls must always be measured. All three oestrogens give similar extinction values. When a fluorimeter was not available (see

later), this method proved reliable for the estimation of pure oestrogens. However, its low degree of specificity, and susceptibility to small quantities of foreign matter, renders it inferior to fluorimetry for general use with tissue extracts.

Purification by paper chromatography

General procedure; apparatus and methods. The following method of paper chromatography was found to give a quick and simple separation, and almost complete purification of the three oestrogens in the tissue extracts. To avoid streaking, and to give reproducible R_F values in the presence of the large amount of greasy impurity found in the extracts, it was found essential to use an elevated temperature under the carefully controlled conditions recently described by Bush (1952) for adrenal steroids. All chromatography tanks were enclosed in a cabinet (dimensions 100 × 70 × 70 cm. high), controlled thermostatically at $32 \pm 0.5^\circ$ (Bush, 1952). Good results have also been obtained with a well-insulated tank placed in a laboratory incubator. The dimensions of the glass tanks were 30 × 20 × 40 cm. high, and to ensure proper equilibration of the highly volatile solvents at the relatively high temperature used, the inner walls of the tank were covered with wads of filter paper. Those at the sides dipped into the mobile phase covering the bottom, while those at the ends dipped into vessels containing 200 ml. of stationary phase (cf. Bush, 1952).

Spreading of the spots due to evaporation of solvent through the lid seal was reduced by covering the tank lid with a piece of cloth, the edges of which hung a few inches down the outside of the tank.

The chromatograms were hung with approx. 6 cm. of paper running with a slope of 1 in 1 between the liquid level and the supporting rod. The starting line was 2 cm. beyond this rod (cf. Block, LeStrange & Zweig, 1952).

Spotting technique. The final extracts in ethanol (Fig. 1), were placed on strips 5 × 18 in. of Whatman no. 541 filter paper, and the ethanol was evaporated by a stream of air directed under the paper. For quantitative work, the paper must previously be extracted with methanol in a Soxhlet apparatus for 48 hr. to reduce the amount of impurities soluble in methanol.

Up to 20 μg . of each oestrogen could be run on the paper before any sign of streaking appeared; the minimum amount for detection is 0.5–1.0 μg . Single preparative runs of extracts from 100 g. of placental tissue have been made, but, for estimations, extracts from 10–20 g. were placed on the paper.

Solvent systems. The mobile phase for oestrone and oestradiol was made from 1000 ml. light petroleum (b.p. 100–120°); the stationary phase from methanol (1000 ml.). For oestriol these were respectively from benzene (1000 ml.) and from a mixture of methanol (500 ml.) and water (500 ml.). The phases were mixed, equilibrated overnight in the cabinet, separated, and placed in their respective compartments in the tanks (except in the running troughs). A store of the equilibrated mixture was kept in the cabinet to replenish the tanks from time to time.

Equilibration of paper. If the tank were opened during insertion of the paper for the minimum time only, not more than 5 min. were needed for equilibration.

Running of the chromatograms. The runs were started by pouring 35 ml. of mobile phase into the troughs by means of a funnel through holes in the lid of the tank (Hanes & Isherwood, 1949). The solvent systems have been so

Fig. 4. Standard curves for oestriol treated with Folin & Ciocalteu's reagent. To oestriol, 0.4 ml. of diluted Folin & Ciocalteu's reagent was added. Tubes were heated in boiling-water bath for 10 min. ○—○ (green colour). To this solution was added 0.15 ml. 25% Na_2CO_3 and the tubes were kept in bath at 25° for 10 min. +—+ (blue colour). All absorptions were measured against blank solutions containing no oestrogen, and all readings were taken in region of max. absorption (690 $\text{m}\mu$.) using 0.5 ml. cells in a Spekker Photoelectric Absorptiometer (filter Ilford 608, transmission max., 680–700 $\text{m}\mu$.).

arranged that the runs may take place overnight. With a 15 hr. run, the spots were well distributed over the papers. The R_F values in the respective systems are oestrone, 0.2; oestradiol-17 β , 0.07; and oestriol, 0.13 (Fig. 5).

Detection of spots

Methods already described. The detection of oestrogens on paper chromatograms has always proved difficult. The iodine technique described by Bush (1950) will detect 1 μ g. of steroid/cm.². The method is however by no means specific, and the spots disappear after a few hours owing to evaporation of the iodine. Boscott (1951) either immersed the paper in, or spotted it with, 90% (v/v) H₂SO₄, and looked for fluorescence under ultraviolet light.

Detection by ultraviolet absorption. A 125w mercury-vapour lamp with filters, Chance OX7; and Cl₂, 1.25m-NiSO₄ and 0.35m-CoSO₄, each contained in 4 cm. diam. quartz flasks, gives sufficient light of the right wavelength (approx. 281 m μ .) to detect 2 μ g. of oestrogen/cm.². The exposure of 30 sec. is made on Kodak Reflex Contact Document Paper placed behind the chromatogram at a distance of 1 m. from the lamp. The specificity is poor, but this method can be used for relatively large amounts of oestrogen, and has the advantage that the oestrogen is left intact for subsequent assay.

Detection by the use of Folin & Ciocalteu's reagent. This reagent gives a sensitive and satisfactory method for the detection of oestrogens on paper chromatograms. It is not necessary to apply heat, and, for the alkali, vapour from

ammonia solution is preferable to a spray of Na₂CO₃, as the latter tends to produce a darker background and spoils the texture of the paper.

The paper was lightly sprayed with Folin & Ciocalteu's reagent (freshly diluted, 1 vol. reagent to 5 vol. water), and placed immediately into a glass tank, the bottom of which was covered with NH₃ soln. (sp.gr. 0.880). When the spots reached maximum intensity (about 5 min.), the paper was removed and dried at room temp. to give a permanently stable chromatogram; the oestrogens showed as blue spots on a pale-blue background (Fig. 5). The minimum amount detectable is 0.5 μ g./cm.². The spray used for applying the reagent must be all of glass or Bakelite construction, as many metals react with the reagent to give a dark-blue background to the chromatogram.

Though the blue colour is formed by many other substances besides phenols, we found that, within the group of steroids investigated, in addition to the oestrogens, only those bearing an α -ketol side chain gave a strong colour (Table 1).

For quantities of oestrogen over 1 μ g. the reaction does not go to completion during a single spraying, consequently, for amounts more than this, the depth of colour on the paper is only quantitative if the paper is put through the complete process (drying after each treatment with NH₃) at least 4 times. The oestrogen content of the spots can then be obtained by visual comparison with standards run in parallel. Alternatively, the amount of oestrogen present may be estimated after the first spraying by cutting the spot into small pieces, adding reagent and treating as previously described under the estimation using Folin & Ciocalteu's reagent.

Fluorimetric estimation

For this method the positions of the spots were found by means of parallel runs with smaller amounts of similar extract, and of pure oestrogens. The strips containing these

Fig. 5. Chromatograms of placental extracts developed in benzene, light petroleum, methanol and water systems: (a) 'free' oestrone and oestradiol from 50 g. placental tissue; (b) 5 μ g. pure oestrone and oestradiol; (c) 'free'-oestriol fraction from 50 g. placenta; (d) 5 μ g. pure oestriol. Spots made visible with Folin and Ciocalteu's reagent.

Table 1. Specificity of steroids to Folin & Ciocalteu's reagent

The steroid was placed on Whatman 541 filter paper in ethanolic solution, the ethanol was allowed to evaporate, the paper was lightly sprayed with Folin & Ciocalteu's reagent diluted with 5 vol. water and immediately placed for 5 min. in the vapour from NH₃ solution, sp.gr. 0.880.

Steroid	Min. amount detectable by spray technique (μ g./cm. ² of paper)
Oestrone	0.5
Oestriol	0.5
Oestradiol-17 β	0.5
17 α -Hydroxy-11-dehydrocorticosterone (cortisone)	2.0
Deoxycorticosterone	5
17 α :21-Dihydroxypregnane-3:11:20-trione	20
21-acetate (4:5-dihydrocortisone acetate)	
17 α -Hydroxy-11-dehydrocorticosterone 21-acetate (cortisone acetate)	50
Dehydroepiandrosterone	50
Androsterone	100
Progesterone	100
3 β :21-Dihydroxypregn-5-en-20-one	100
21-acetate (21-acetoxypregnenolone)	
Pregnane-3 α :20 α -diol	100

parallel runs were cut out and sprayed with Folin & Ciocalteu's reagent. The smallest area of paper ensuring removal of all oestrogen in the main runs was then cut out, together with similar areas from a blank part of the chromatogram. Standard amounts of oestrogen were placed on these blank papers.

Despite pre-extraction with methanol for 48 hr., it was found necessary to extract each paper with the same amount of solvent for a standard time, and for this purpose an apparatus was constructed which dripped 5 ml. of methanol from capillary tubes over a period of 8 min. The drops were allowed to fall on the top corners of the papers, each suspended on a stainless-steel hook, and collected from the bottom corners into the test tubes in which the fluorescence was to be developed. The methanol was removed by a stream of N_2 with the tubes immersed in a water bath at 70°.

Several methods for the fluorimetric determination of the oestrogens have been described (Finkelstein, Hestrin & Koch, 1947; Jailer, 1948; Bates & Cohen, 1950; Engel *et al.* 1950; Diczfalussy, 1953). Each of these techniques was investigated, and that of Diczfalussy (1953) found to be the most satisfactory for general use. In this method the oestrogen is dissolved in 0.5 ml. ethanol and the fluorescence developed by heating for 10 min. in a boiling-water bath with 8 ml. of 88% (v/v) H_2SO_4 followed by cooling in cracked ice.

A sensitive fluorimeter, suitable for estimating the fluorescence produced by amounts down to 0.1 $\mu g.$ oestrogen, which has given satisfaction during 3 yr. general use, was constructed from readily available materials utilizing the photomultiplier circuit described by Lowry (1947). With Chance O.N. 20 (heat) and Chance O.B. 10 (transmission max. 425 $m\mu$.) as primary filters, and Ilford Spectrum Green 604 (transmission max. 520 $m\mu$.) as secondary filter, the sensitivity of the instrument was such that a solution of 0.05 $\mu g.$ sodium fluoresceinate/ml. gave a current of 0.5 μA and half-scale deflexion (8 cm.) on the galvanometer.

The development of the fluorescence is very susceptible to external influences, but the work of Braunsberg (1952), Brown (1952) and of Diczfalussy (1953), has shown that if these influences are controlled, good results can be obtained. This control entails completing a standard graph with each set of estimations, because we have found, in agreement with Braunsberg (personal communication), that graphs produced at different times may vary considerably.

Final identification of oestrogens from placenta

The extracts from 160 g. of placental tissue ('free' fraction Fig. 1) were run on chromatograms. The spots were removed from the paper and eluted as above. The methanol was evaporated, the residue dissolved in 1 ml. of ethanol and the ultraviolet absorption spectra were measured and compared with those for pure oestrogens (Fig. 6).

It was found that the filter paper, even after previous extraction in the Soxhlet extractor for several days, still contained a substance which was extracted by methanol and absorbed strongly in the ultraviolet region, causing distortion of the oestrogen curve. Extracts were therefore made of similar blank areas of filter paper and used as the solvent blank for obtaining the absorption curves.

Fig. 6. Absorption spectra of oestrogen spots eluted from chromatograms and compared with pure oestrone. 1, 30 $\mu g.$ pure oestrone/ml. ethanol; 2, oestradiol; 3, oestriol and 4, oestrone from 160 g. placental tissue, dissolved in 1 ml. ethanol. The absorptions were measured against extractions from similar areas of blank chromatogram paper. The absorption curves for pure oestriol and oestradiol are similar to that for oestrone. Readings were taken at every $m\mu$. over the whole range shown and the curves are drawn through the experimental points.

Table 2. *Comparison of oestrogen in paper-chromatogram spots from 400 g. placental tissue when assayed by various methods*

Oestrogens were extracted and purified by separation in solvents and the final products were chromatographed in benzene, light petroleum, methanol and water systems as described in the text.

Method of assay	Oestriol ($\mu g./kg.$)	Oestrone ($\mu g./kg.$)	Oestradiol ($\mu g./kg.$)	Estimated error of method (%)
Visual comparison of spots obtained with Folin & Ciocalteu's reagent	40	16	4	± 10
Absorption in ultraviolet (with allowance for estimated impurity)	40	25	4	± 20
Biological (cornification of mouse vagina)	25-60*	17	1.4*	± 10
Fluorimetry	40.5	13.8	5.0	± 5

* The amounts of material available for assay were insufficient for more accurate estimations.

Although peaks were found for oestriol and oestrone, the oestradiol content of the placental extracts was always low, and in this case was insufficient to produce a clear peak at 281 m μ .

The ethanol was then removed from these solutions, and the residue coupled with Fast Black Salt K, followed by chromatography (Heftmann, 1950). In each case well-defined spots were produced, identical in all ways with spots produced by parallel runs of the three pure oestrogens.

The absorption curves were obtained for similar eluates which were then assayed biologically (by vaginal cornification produced by injection into adult, spayed female mice) and fluorimetrically, with the results shown in Table 2.

Thus, in addition to the spots having the same R_F values as pure oestriol, oestrone and oestradiol, their identity has been proved by ultraviolet absorption, chromatography of the product obtained by coupling with azo dye, development of fluorescence in H_2SO_4 and by bio-assay, and good agreement of results has been obtained by the various methods of estimation (Table 2).

Recovery experiments

As preliminary work showed there was a considerable loss of the oestrogens during purification, the recovery of the three oestrogens added to full-term placental tissue was investigated. The importance of the rigorous purification of all solvents, the necessity of completing all processes above room temperature under N_2 , and the need to keep extracts in the deep freeze (temp. -20°) whenever possible was proved early in the investigation. These precautions, however, did not prevent a large loss, and to arrive at the true level of oestrogen in the samples of placental tissue, it was necessary to complete several large-scale recovery experiments (Table 3).

The oestrogens were added in ethanolic solution after the first addition of 80% (v/v) ethanol to the tissue (see Fig. 1), and, as Diczfalussy (1953) has also shown, the added oestrogen did not appear in the 'conjugated' or 'protein-bound' fractions. Heating the tissue to 100° in N_2 before adding the oestrogen increased the recovery in all cases, whilst heating in air increased it further, but destroyed some of the original

oestrogen. The use of thiourea did not improve the recovery, though Bauld (1953) found that some antioxidants prevent losses at certain stages in the extraction of oestrogens from urine.

Though recoveries varied from batch to batch, the good agreement of duplicate estimations carried out on the same specimen (Table 4), shows that the losses involved in purification were constant for each batch of minced placenta.

Very little loss took place during chromatography and elution from the chromatogram. Oestrogens added to the extract placed on the paper were recovered as follows: oestriol, 97%; oestrone, 95%; oestradiol, 84%.

The experimentally determined values of free oestrogen per kg. in seven full-term placentae were: oestriol, 190 μ g.; oestrone, 86 μ g.; oestradiol, 25 μ g. When these values are corrected for the very low recoveries found in the appropriate experiments (see Table 4), the estimated values are: oestriol, 680 μ g.; oestrone, 580 μ g.; oestradiol, 170 μ g. Since these estimated values are based on very low recoveries, and since those recoveries vary widely from one sample to another (for example, between 18.5 and 49.4% for oestriol, and between 4.5 and 20% for oestrone), the authors wish to emphasize that the recovery figures may only be very roughly applicable to other samples of placental tissue.

The corrected amounts lie between 4 and 16 times more than was reported by Huffman, Thayer & Doisy (1940), working with more than 400 kg. of placental tissue. They found respectively 140, 35 and 38 μ g./kg. of each oestrogen. Diczfalussy (1953) found the amounts of the free oestrogens to be respectively 125.4, 46.7 and 3.1 μ g./kg. Neither of these figures corrected for losses during purification.

As recovery experiments were not carried out on the 'conjugated' and 'protein-bound' fractions, losses in purification cannot be applied to the average results for these fractions shown in Table 4.

To ascertain how much of the oestrogen in the placental tissue was due to the small amount of retained blood, estimations were carried out on 10 ml. samples of venous blood taken just before or after delivery. The sensitivity of the method was not sufficient to give accurate results on such small amounts of material, but was sufficient to show

Table 3. *The recovery of 'free' oestriol, oestrone and oestradiol added to full-term human placental tissue*

The pure oestrogen was added to the mince in ethanolic solution immediately after the addition of 80% (v/v) ethanol for extraction.

			Recovery of added 'free' oestrogen					
Expt. no.	No. of placentae pooled	Treatment of placentae before extraction	Oestriol		Oestrone		Oestradiol	
			Amount added (μ g.)	Amount recovered (%)	Amount added (μ g.)	Amount recovered (%)	Amount added (μ g.)	Amount recovered (%)
1	3	None	500*	22.6	500*	18.0	500*	11.4
		None	30*	19.4	30*	16.0	30*	12.0
		Heated 25 min. at 100° in N ₂	500*	37.7	500*	37.2	500*	20.4
			30*	68.0	30*	32.6	30*	18.0
2	2	None	500†	49.4	500†	20.0	500†	13.5
		None	30†	37.3	30†	14.3	30†	17.3
5	2	None	20*	18.5	20*	4.5	20*	15.0
		Heated 25 min. at 100° in air	20*	99.0	20*	13.0	20*	70.0
Average recovery from untreated placentae				26.7		13.5		13.7

* Added to 150 g. placental tissue.

† Added to 195 g. placental tissue.

Table 4. *Experimental values for 'free', 'conjugated' and 'protein-bound' oestriol, oestrone and oestradiol, with values corrected by recovery experiments (cf. Table 3) for the 'free' fraction with the same placentae*

For details see text.

Expt. no.	No. of placentae pooled	Treatment of placentae before extraction	'Free' oestrogen isolated ($\mu\text{g./kg.}$)			'Conjugated' oestrogen isolated ($\mu\text{g./kg.}$)			'Protein-bound' oestrogen isolated ($\mu\text{g./kg.}$)			'Free' oestrogen content corrected on basis of recovery expt. ($\mu\text{g./kg.}$)		
			Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol
			($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)	($\mu\text{g./kg.}$)
1	3	None	157	108	0	65	25	0	33	16	0	748	635	0
		Heated 25 min. at 100° in N_2	168	111	0	68	23	0	31	16	0	317	317	0
2	2	None	205	112	14	—	—	—	—	—	—	416	560	106
		None	234	106	12	—	—	—	—	—	—	627	741	72
3	1	None	300	109	24	61	33	13	14	27	23	—	—	—
		None	300	100	24	79	34	14	39	27	24	—	—	—
4	2	Heated 25 min. at 100° in N_2 + thiourea to 0.1%	288	194	10	—	—	—	—	—	—	—	—	—
		Heated 25 min. at 100° in N_2	275	194	8	—	—	—	—	—	—	—	—	—
5	2	None	148	20	76	0	8	17	0	5	10	730	440	507
		Heated 25 min. at 100° in air	92	19	57	0	8	8	0	1	17	93	146	82
Average content of untreated placentae			190	86	25	44	21	8	21	14	7	678	584	170

that the maternal blood contains considerably less free oestrogen (10–50 $\mu\text{g.}$ of each oestrogen/kg.) than was found in placentae. This is in agreement with the values reported by Diczfalusy (1953) and Veldhuis (1953).

The following procedure was developed to find the approximate amounts of maternal and foetal blood in placental tissue. The maternal blood in the sinuses was obtained by washing the placenta (approx. 300 g.) 10 times, with gentle massage, in 30 ml. quantities of water. The placenta was then finely minced and extracted 10 times with 50 ml. quantities of water. Haemoglobin was measured in the washings by the method of Haldane (1901). Haemoglobin estimations were also carried out by the method of Clegg & King (1942) on the mother and baby soon after delivery. The results show that the tissue contained approximately 6% foetal, and 3% maternal blood. Assuming that the foetal-blood oestrogen is not higher than the level in maternal blood, the blood content of the placental tissue could not account for more than 5 $\mu\text{g./kg.}$ of each oestrogen. This (uncorrected) content is small compared with the amounts found in placenta.

DISCUSSION

By the use of paper chromatography, two of the great technical difficulties involved in the chemical estimation of the minute quantities of oestrogens in tissue have been largely solved; namely the complete separation of the three oestrogens, and their preparation in a sufficient state of purity. This is shown by the concordant results obtained by the four widely different methods of estimation given in Table 2.

Some of the possible errors and losses at various stages of purification have been extensively discussed by Diczfalusy (1953). It is difficult to see how these can be further eliminated except perhaps by the use of suitable antioxidants, or of labelled oestrogens for use as internal standards to measure the losses. However these are not yet readily available.

The corrected oestrogen contents of full-term placentae present quite a different picture from that shown by the previously reported uncorrected values (Huffman *et al.* 1940; Diczfalusy, 1953) which suggested that there was more than 3 times as much oestriol as oestrone or oestradiol. The corrected figures show that oestrone is present in similar amount to oestriol, and that there is much more oestradiol than previously suspected.

Recently, Ryan & Engel (1953*b*) have shown that sliced, full-term placental tissue, when incubated separately with relatively large amounts of each of the three oestrogens, converts oestradiol into oestrone and degrades both to unrecognizable metabolites. Oestriol was recovered almost quantitatively after incubation. It is possible that some conversion and destruction is taking place during the interval between delivery and extraction of the tissue, so the original level of oestradiol may be higher when the placenta is *in situ*.

The advantages of the method described in this paper, and also that of Diczfalusy (1953), are that they require very much smaller amounts of material and take far less time than the earlier procedures (cf. Huffman *et al.* 1940). The method is being applied to the separation and estimation of oestrogens in urine.

SUMMARY

1. An investigation has been made into many of the chromatographic techniques now available, with a view to their use in the separation and purification of tissue oestriol, oestrone and oestradiol.

2. A method has been developed for the isolation and estimation of these three oestrogens in placental tissue. It involves a preliminary chemical separation followed by paper chromatography, elution and fluorimetric estimation.

3. The use of Folin & Ciocalteu's reagent in a sensitive method for detection of the oestrogens on paper chromatograms is described.

4. The use of azo-dye coupling followed by paper chromatography (Heftmann, 1950), has been adapted to the quantitative assay of oestrone and oestradiol, and a new method of assay for oestriol, oestrone and oestradiol using Folin & Ciocalteu's reagent is described.

5. The uncorrected average quantities of 'free' oestrogen/kg. in seven full-term placentae were: oestriol, 190 $\mu\text{g.}$, oestrone, 80 $\mu\text{g.}$ and oestradiol, 25 $\mu\text{g.}$ The values for 'conjugated' oestrogen were respectively: 44, 21 and 8 $\mu\text{g.}$, and for 'protein-bound' oestrogen: 21, 14 and 7 $\mu\text{g.}$ Large differences were found in the oestrogen content of individual placentae.

6. Recovery experiments show that the normal methods of extraction and purification, when applied to the 'free' oestrogens in full-term placental tissue give losses of between 70 and 90%. The corrected average quantities of 'free' oestrogen/kg. in seven full-term placentae were estimated as: oestriol, 680 $\mu\text{g.}$, oestrone, 580 $\mu\text{g.}$ and oestradiol, 170 $\mu\text{g./kg.}$ Whilst these corrections are accurate for the seven placentae examined, they may be only roughly applicable to other samples of this tissue.

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