THE ESTIMATION OF OESTRIOL, OESTRONE AND OESTRADIOL-17β IN HUMAN BLOOD

by

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Contents

I. INTRODUCTION

The Natural Oestrogens

The ovary secretes two classes of hormones, oestrogens and progesterone, which play important roles in sexual development and reproduction in the female. These hormones are also produced in relatively large quantities by the human placenta. A reliable method for detecting and measuring oestrogens was first developed in 1923 by Allen and Doisy. This was based upon the ability of these hormones to produce an oestrus response when injected into ovariectomised mice or rats. This response is characterised by cornification of the epithelium of the uterus after injection of oestrogenic material. Using this method Ascheim and Zondek (1927) detected the presence of large quantities of oestrogenic material in the urine of pregnant women and suggested that it was secreted by the placenta. Urine of pregnant women has proved to be an excellent source of oestrogens and up to the present time twelve oestrogenic compounds have been isolated from this source. Doisy (1929) and Butenandt (1929) independently isolated oestrone from this source and soon afterwards Marrian (1930) isolated oestriol from the same source. In early experiments with these compounds, a dihydro derivative of oestrone, i.e. oestradiol, was found to have an even higher biological activity than oestrone, which was at that time the most biologically active oestrogen, and this was subsequently isolated from sow ovaries by MacCorquodale, Thayer and Doisy (1935). Oestradiol-17β* was later isolated from the urine of

* Throughout this thesis the term oestradiol will refer to oestradiol-17β.

pregnant women by Huffman, MacCorquodale, Thayer, Doisy, Smith & Smith (1940).

Until 1955 these were the only oestrogens isolated from human urine. With the advent of more sensitive and reliable methods of detection, several new oestrogens have been isolated either from the urine of pregnant women or from the urine of individuals to whom 16-C^{14} oestradiol-17β has been administered. The following compounds have been isolated and identified: 16-epioestriol (Marrian & Bauld, 1955); 16α-hydroxyoestrone (Marrian, Watson & Panatonni, 1957; Marrian, Loke, Watson & Panatonni, 1957); 18-hydroxyoestrone (Loke, Watson & Marrian, 1957; Loke, Marrian, Johnsen, Meyer & Cameron, 1958); 16β-hydroxyoestrone (Brown, Fishman, Gallagher, 1958; Layne & Marrian, 1958); 2-methoxyoestrone (Kraychy & Gallagher, 1957a, b; Loke & Marrian, 1958); 16-oxo-oestradiol (Levitz, Spitzer & Twombly, 1956; Layne & Marrian, 1958); 2-methoxyoestriol (Fishman & Gallagher, 1958); 2-methoxyoestradiol (Frandsen, 1958); 17-epioestriol and 16, 17-epioestriol (Breuer & Pangels, 1961) and 2-hydroxyoestrone (Fishman, Cox & Gallagher, 1960).

Many methods have now been proposed for the measurement of oestrogens in biological fluids. At first biological methods were used, but these have gradually been superseded by more convenient chemical methods. The first chemical methods were limited to measuring the relatively large amounts (mgms.) of oestrogens present in pregnancy urine, but the sensitivity of the chemical methods has recently been increased so that they are applicable also to the relatively small amounts (μg.) in urine of non-pregnant subjects;

an increase in sensitivity of approximately 1000-fold. The present work is concerned with increasing the sensitivity of one chemical method still further to the measurement of fractions of a microgram of oestriol, oestrone and oestradiol in extracts of blood taken from pregnant women.

Methods of measurement of oestriol, oestrone and oestradiol.

1. Bioassay

Bioassay has been widely used as a means of measurement of oestrogens. The oestrogenic response depends to a certain extent on the design of the assay, e.g. the mode of administration and the number of injections (Thayer, Doisy & Doisy, 1944), and on the species of animal used. In addition, the three oestrogens have markedly different potencies, oestradiol being the most active and oestriol the least active. Impurities in the extracts can have marked depressing or potentiating effects (Emmens, 1939), therefore the extracts require careful purification and separation into the various oestrogen fractions. The most frequently used biological assay is that described by Allen & Doisy (1923). This assay measures the degree of vaginal cornification after injection of oestrogens into ovariectomised mice and rats. Another method of assay is the measurement of the increase in uterine weight which follows the administration of oestrogens to immature rats (Astwood, 1938). Bioassays have a low degree of precision and do not give satisfactory quantitative results. They are more commonly used now as checks on the specificity of a chemical method, rather than as quantitative methods of determination.

2. Kober reaction.

Kober (1931) observed that the yellow colour which developed when oestrogens were heated with sulphuric acid gave rise to a deep red colour on dilution with water and further heating. Phenol enhanced this red colour and quenched the fluorescence which was also present. As described by Kober, the production of a pink colour with a maximum in the region 505-520 mμ. is highly specific for natural oestrogens. The original Kober reaction did not give a stable and reproducible colour, and was unsatisfactory for the measurement of oestrogens. Consequently many modifications have been proposed; for example, see Cohen & Marrian (1934), Venning, Evelyn, Harkness and Browne (1937), Stevenson & Marrian (1947).

Brown (1952) and Bauld (1954) made an intensive study of the conditions necessary for the production of the maximum amount of colour and also conditions which gave a stable colour. They found that reducing agents were important in both stages of the reaction and that each of the three oestrogens required a different sulphuric acid concentration to give the optimum results. For example, when using quinol as the reducing agent, the optimum sulphuric acid concentrations in the first stage were 76% H_2SO_4 for oestriol, 66% H_2SO_4 for oestrone, and 60% H_2SO_4 for oestradiol. Further dilution with water and heating were necessary in the second stage for complete formation of the red colour. Maximum intensity and stability were obtained when the final sulphuric acid concentration was 50-60%. More dilute concentrations caused the colour to fade. Quinol was found to be the best reducing

agent, and the reagents used contained 2% quinol (w/v). The Kober reaction as described by these workers could be used to measure 5-10 µg. oestrogen in a final volume of 3-4 ml.

Both workers applied the Allen correction formula (Allen, 1950) to correct for the optical densities of the impurities present. Impurities present in urine extracts give rise to yellow-brown colours in the Kober reaction. These can be corrected for either spectrophotometrically (Venning et al. 1937; Allen, 1950) or by destruction of the Kober colour by heating or by the addition of hydrogen peroxide after measurement of the colour given by oestrogens and impurities (Cohen & Marrian, 1934; Stevenson & Marrian, 1947). The Allen correction formula, used by Brown and Bauld, is now the most commonly used method of correction. It depends on the assumption that the wave-length absorption curves of colours due to impurities in the urine extracts are linear in the range of the absorption maximum of the oestrogen colour. This is difficult to prove for all urine extracts, but considerable evidence has been accumulated to show that this assumption is valid for the urine extracts prepared by the methods of Brown (1955) and Bauld (1956).

Ittrich (1958) has recently described a method for the extraction of the Kober colour with chloroform containing 2% p-nitrophenol (with 1% ethanol to dissolve the nitrophenol). He used a one-stage Kober reaction in which the oestrogens were heated with a reagent containing 68% H_2SO_4, 2% quinol for oestriol and 52% H_2SO_4, 2% quinol for oestrone and oestradiol; after cooling, the sulphuric acid mixture

was diluted with an equal volume of water to a final sulphuric acid concentration of about 30%, which was not critical. The Kober colour was then extracted with an equal volume of chloroform-p-nitrophenol reagent. The yellow-brown impurities remained in the aqueous phase which was discarded, and the optical density of the red colour extracted by the chloroform was measured at the wavelengths 508, 539 and 570 mμ. The Allen correction formula was applied to calculate the true optical density of the oestrogens present. This procedure resulted in extensive purification of the Kober colour, the absorption maximum was shifted from 516 mu to 539 mμ, and the absorption curve was narrowed and increased in height. The Ittrich reaction is now being investigated in several laboratories and is proving extremely valuable in determining small quantities of oestrogens in urine and blood (Ittrich, 1960; Salonkangas & Bulbrook, 1961; Personal communication, Roy & Brown). However, this is a recent development, and the method to be described in this thesis was fully developed before Ittrich reported his modification.

3. Fluorescence reactions.

Wieland, Straub and Dorfmuller (1929) and Marrian (1930) noted that intense fluorescence developed when oestrogens were heated with sulphuric acid. Other agents, such as phosphoric acid, have the same effect. This fluorescence reaction is extremely sensitive and has been widely used for the fluorimetric measurement of oestrogens (Finkelstein, Hestrin & Koch, 1947; Bates & Cohen, 1947; Jailer, 1947; Engel, 1950).

Maximum fluorescence of the oestrogens is produced by a blue incident light of approximately 420 mμ; the emitted light is greenish-yellow with a broad spectrum showing a maximum at approximately 525 mμ. Careful selection of filters to prevent transmission of any reflected primary light through the secondary filter is necessary.

Many substances other than oestrogens fluoresce with sulphuric or phosphoric acid, and the fluorescence of the oestrogens can be quenched (Veldhuis, 1953) or enhanced (Bates & Cohen, 1950) by impurities present in the extracts.

Veldhuis (1953) and Braunsberg, Osborne & Stern (1954) attempted to assess the effects of the impurities by measuring the fluorescence produced by the extract and comparing it with the fluorescence produced by a known amount of pure oestrogen added to another portion of the same extract.

The fluorescent compound can be extracted with chloroform-p-nitrophenol reagent (Ittrich, 1958), when the absorption maximum shifts to 545 mμ and the maximum of emitted light is 557 mμ. The Ittrich method appears to give the specificity of the Kober reaction, with the sensitivity of the fluorescence reactions, but was only recently reported.

Measurement of urinary oestrogens.

Urine extracts have to be extensively purified before the oestrogens present can be determined accurately by the Kober or fluorescence reactions. In addition the three oestrogens must be separated, because they do not all give the same intensity of colour or fluorescence under the same conditions.

Urinary oestrogens are conjugated with glucuronic acid, and possibly with sulphuric acid. These conjugates can be extracted from urine with solvents such as butanol, but in general they are first hydrolysed by boiling with acid or by incubation with preparations containing the enzyme β-glucuronidase. Oestriol, oestrone and oestradiol are stable to boiling with 15 vol. % concentrated HCl. This is a satisfactory method for hydrolysis of oestriol, oestrone and oestradiol conjugates, but losses occur due to the combined action of a normal urinary constituent and boiling HCl (Brown & Blair, 1958). No losses occur during enzymic hydrolysis, which gives results nearer to the true result, but prolonged incubation may be necessary for complete hydrolysis, and the method is not as convenient as acid hydrolysis for routine day to day use.

The free oestrogens are then extracted from the hydrolysed urine with ether. Advantage is taken of the phenolic properties of the oestrogens and the ether extracts are washed with sodium bicarbonate or sodium carbonate solutions to remove the acidic fraction. This removes much of the pigment from the extracts.

The extracts can be purified in several ways. Oestriol is much more hydrophilic than oestrone and oestradiol and is readily extracted from benzene with water, while dilute alkali subsequently extracts the oestrone and oestradiol. The three oestrogens can be separated and purified by counter-current distribution (Engel, Slaunwhite, Carter & Nathanson, 1950; Diczfalusy & Lindkvist, 1958), by adsorption chromatography of the methyl ethers (Brown, 1955), or by partition chromatography on

Celite columns (Bauld, 1955; Aitken & Preedy, 1956). The oestrogen content is then determined by the Kober or fluorescence reactions.

Brown (1955) and Bauld (1956) published chemical methods for the determination of urinary oestrogens based on the Kober colour reactions. Both methods involved hydrolysis of the urinary conjugates by boiling with 15 vol.% concentrated HCl, and ether extraction of the free oestrogens. The ether extracts were washed with sodium bicarbonate solutions of pH 10.5 to remove pigments. In the method of Brown, the residue from the ether extracts was dissolved in ethanol, and benzene and light petroleum were added. Oestriol was first extracted with water, and oestrone/oestradiol with 1.6% NaOH. These two fractions were methylated in borate buffer with dimethyl sulphate. The methylated fractions were further purified and separated into oestriol, oestrone and oestradiol methyl ether fractions by adsorption chromatography on standardised deactivated alumina. The oestrogen content of the 3 fractions was then determined colorimetrically by the Kober reaction.

In Bauld's method the residue was dissolved in ethanol/benzene, and the oestriol extracted with water; the extract was made normal to NaOH and refluxed 30 minutes; the oestriol was then extracted with ether; the ether was washed with $NaHCO_3$ and water, and the ether was distilled. The oestriol was finally purified by partition chromatography on a Celite column. After chromatography the separated oestrone and oestradiol fractions were refluxed 30 minutes

with NaOH, and then re-extracted with benzene which was washed with sodium carbonate solution and water. The concentrations of the three oestrogens were then measured by the Kober reaction.

The reliability of both these methods have been described (Marrian, 1956). The recovery of oestrogens added to hydrolysed urine, which provides valuable information concerning the accuracy of the method excluding hydrolysis, is of the order of 80-90%. Recovery of oestrogens added to urine before acid hydrolysis is 60-70%, showing that there is a further loss of 10-12% oestrogens during hydrolysis.

The specificity of the Brown method was based on a) the high specificity of the Kober reaction, b) the purification procedures employed, and c) the chromatographic behaviour of the methylated oestrogen fractions on alumina columns. This was later supported by counter-current distribution studies (Diczfalusy, 1955), comparisons with bioassay (Bulbrook, Greenwood & Williams, 1957), and isotope dilution (Gallagher, Kraychy, Fishman, Brown & Marrian, 1958). The specificity of the Bauld method depended on a) the high specificity of the Kober reaction, and b) the behaviour of the urinary oestrogen fractions on partition chromatography columns. When parallel assays were done by both methods on a series of urines, there was very close agreement between results (Marrian, 1955).

Aitken & Preedy (1956) described a method involving gradient elution partition chromatography, and fluorescence with sulphuric acid. The method was a development of the procedure described by Engel et.al. (1950). Urine was refluxed 30 minutes with 1.8N H_2SO_4 and was extracted with ether. The ether was washed with $NaHCO_3$ solution and evaporated to dryness. The residue was partitioned between toluene and N NaOH. The NaOH layer was neutralised to pH 9 and extracted with ether. The residue from the ether extract was subjected to partition chromatography on a Celite column and the eluate was collected in 0.5 ml. fractions. The solvent was evaporated, and the oestrogens determined by the fluorescence with H_2SO_4. The fluorescence intensity was plotted against the tube number and the amount of oestrogen calculated by reference to the curve obtained with pure oestrogens. The specificity of this method was based on the fractional elution method employed and the accuracy as determined by recovery figures was approximately 80%.

Measurement of oestrogens in blood.

Mühlbock (1939) was one of the first to attempt to measure the concentration of oestrogens in blood. He collected blood into alcohol, evaporated the alcohol, and dissolved the residue in benzene. Also after evaporation of the alcohol, the conjugated oestrogens were hydrolysed with acid. The extracts were then assayed biologically. This method indicated that 50% of the oestrogens in blood were in the free form, the remainder being conjugated.

Szego & Roberts (1946) made further studies on this problem. These workers extracted blood, which had been taken from pregnant women, with acetone and hydrolysed the acetone extracts and the protein precipitate with either acid or alkali. Following further processing the oestrogens were asayed biologically and they found that two-thirds of the oestrogen was associated with the plasma proteins and could be removed by alkaline denaturation, and that vigorous acid hydrolysis was unnecessary.

In the last seven years several other methods have been published, notably those of Veldhuis (1953), Preedy & Aitken (1957), Diczfalusy & Magnusson (1958), and Svendsen (1960). The main outlines of these and some of the earlier methods are given in Table 1.

The methods of Veldhuis and of Varangot et al. are relatively simple and rapid, but tend to be non-specific because of fluorescence given by impurities in the extracts. Varangot et al. make no attempt to correct for this. Veldhuis attempts to correct for quenching by impurities by adding a known amount of standard to an aliquot of the extract, and for non-specific fluorescence by destroying

TABLE 1

METHODS FOR THE MEASUREMENT OF

Reference	Hydrolysed with:	Extracted with:	Purification Procedure
Mühlbock (1939)	a) - b) acid	alcohol alcohol	alcohol residue dissolved in benzene
Rakoff et al. (1943)	a) - b) acid	ethanol-ether	ether-alcohol residue dissolved in corn oil
Szego & Roberts (1946)	a) acid, or b) alkali, after extraction with acetone	ether ether	
Veldhuis (1953)	-	ether	partitions between various pairs of solvents such as benzene/Na_2HPO_4 and ether/NaOH
Varangot et al. (1955)	acid	ether	partition between aqueous solution pH 8-8.5/ether and Na_2HPO_4/ether
Preedy & Aitken (1957)	acid	ether	gradient elution chromatography on Celite
Oertel et al. (1959)	acid	ether	counter-current distribution; paper chromatography.
Svendsen (1960)	-	chloroform	partition between aqueous EtOH/pentane; Cl_4/$CHCl_3$ (5/1) water; esterification with S^{35}-pipsyl chloride, addition of I^{131}-pipsyl esters, paper chromatography of pipsyl esters.
*Diczfalusy et al. (1958)	acid, after alcohol extraction	ether, then toluene	methylation; adsorption chromatography on alumina.

*Cord blood used.

TABLE 1

OESTROGENS IN BLOOD

Final assay	Method of correcting for impurities present	Results µg/100 ml.		
biological	-	50% free 138 M.U.†		
biological	-	50% free 179 M.U.†		
biological	-	0.6, 1.8 66% protein-bound.		
fluorescence with sulphuric acid	addition of standards to aliquot of extract to correct for quenching; subsequent quenching of oestrogen fluorescence by H_2O_2 to give a measure of fluorescence of impurities	A 1.0-1.6µg.	B + C 2.1-3.3µg.	
fluorescence with sulphuric acid	-	24-280	11-155	
fluorescence with sulphuric acid	fluorescence of each ml. eluate measured; allowance made for impurities from elution pattern of extract and standard	A 4.3-19.8	B 2.7-17.8	C 0.6-3.7
Kober reaction	Allen correction formula	2.9	5.2	3.3
measurement of S^{35}/I^{131} ratio	-	-	0.1-1	0.2-3
Kober reaction	Allen correction formula	39-68	0-2.3	0.1-1.3

† 1 MU = 0.04-0.1 µg. oestrone.

A = oestriol
B = oestrone
C = oestradiol.

that portion of the fluorescence given by the oestrogens by the addition of a few drops of hydrogen peroxide after reading the fluorescence of oestrogen plus impurities. The method of Preedy & Aitken derives its specificity from fractional chromatography, involving the analysis of large numbers of eluates from the columns. Of the chemical methods published, this is the most reliable, but it is extremely laborious.

Recently Svendsen (1960) described a method for measuring oestrone and oestradiol in blood depending on the formation of their pipsyl esters with S^{35} labelled pipsyl chloride (p-iodobenzenesulphonyl chloride). Specificity depends on the addition of carrier pipsyl ester labelled with I^{131} and purification of the esters by paper chromatography to constant S^{35}/I^{131} ratio. This method can be made extremely sensitive by using compounds of high specific radio-activity and has great possibilities for the future.

Oertel, West & Eik-Nes (1959) isolated oestriol, oestrone and oestradiol from a pool of late pregnancy plasma by an exhaustive process which included acid hydrolysis, counter-current distribution, paper chromatography, and measurement by the Kober reaction. The three oestrogens were finally identified by infra-red spectroscopy. The amounts found in 360 ml. plasma were 10.4 µg. oestriol, 18.6 µg. oestrone and 11.8 µg. oestradiol, corresponding to 3 µg. oestriol, 5 µg. oestrone and 3 µg. oestradiol per 100 ml. plasma.

In contrast with urinary oestrogens, which are conjugated with glucuronic acid and probably with sulphuric acid, blood oestrogens

circulate in a variety of forms. These are arbitrarily defined according to their ease of extraction with organic solvents: e.g. free oestrogens are extracted from aqueous solutions with ether or alcohol; conjugated oestrogens are extracted from aqueous solutions with alcohol but not with ether until they have been hydrolysed to the free form by enzymes or by boiling with acid; protein-bound oestrogens cannot be extracted from plasma proteins with alcohol unless the proteins are denatured under extreme conditions such as by boiling with acid or treatment with alkali (Szego & Roberts, 1946).

Rakoff, Paschkiss & Cantarrow (1943) determined the oestrogen content of late pregnancy blood by direct biological assay of the serum and by assaying ethanol-ether extracts of plasma which had been hydrolysed by treatment with HCl at pH 1 for 6 hours in a boiling water-bath. They also assayed the protein precipitate. By this method they found that that the ether-ethanol extracts gave the same results as direct assay of the serum, but that almost twice as much oestrogen could be extracted from the protein fraction after acid hydrolysis. They showed that a second hydrolysis with acid at pH 1 for 6 hours increased the yield of oestrogen from the protein fraction. On the other hand, Szego & Roberts (1946, 1947) also using biological assays, showed that only one-third of the oestrogens could be extracted from plasma with acetone, the remaining two-thirds could be extracted after alkaline denaturation of the proteins. They suggested that the more vigorous conditions of acid hydrolysis were unnecessary. In addition, they showed that all the oestrogens could be removed from plasma by dialysis through Visking membrane. In recent years other workers have

used dialysis as a means of splitting steroid-protein complexes. Slaunwhite & Sandberg (1959) showed that 77% of oestriol-16-C^{14} added *in vitro* to plasma from a non-pregnant woman was freely dialysable through a Visking membrane against saline, but not against serum albumin. Our present knowledge concerning the exact nature of the various forms of oestrogens circulating in blood and their quantitative interrelationships and significance is far from complete and very much more work is required on these points.

Aims of the present investigations.

The aim of the present work was to determine whether assays of blood oestrogens could aid in the clinical assessment of pregnant women, and whether they could also be used to study some physiological problems during pregnancy. The possibility of measuring blood oestrogens in men and non-pregnant women was not considered at this stage because of the very low concentrations which were likely to be present.

A method was therefore required which was suitable for the routine determination of oestrogens in large numbers of blood samples and which was sensitive enough for use with a convenient volume of, say, 10 ml. of blood. Biological methods lacked precision and were laborious. The existing chemical methods for measuring oestrogens in blood were either non-specific (Veldhuis, 1953; Varangot *et al.*,1955) or too laborious for routine use (Preedy & Aitken, 1957). The method of Veldhuis was rapid and convenient, but its specificity was based on assumptions (see Table 1), the validity of which was difficult to prove. Varangot *et al.* (1955, 1956, 1957) ignored the interference

by impurities in the fluorescence reaction, and depended only on the partitions used in the method for the specificity of the method. Their results were obviously too high when compared with those obtained by more reliable methods.

The method of Preedy & Aitken (1957) for blood, which was published several years after the commencement of the present project, depended for specificity on gradient elution partition chromatography of the extracts (see Table 1). By this means the fluorescence given by the impurities could be rejected and considerable confidence placed on the results obtained. However, this procedure is too laborious for the application planned here.

A method, based on the more convenient procedure described by Brown (1955) for estimating oestriol, oestrone and oestradiol in human urine, seemed a distinct possibility for the routine determination of oestrogens in blood. From preliminary studies it seemed that the procedure required should be capable of measuring as little as 0.05 µg. oestrogen in 10 ml. blood. The existing fluorimetric methods are sensitive enough for measuring these small amounts and consequently were applied to the estimation of oestrogens in blood extracts prepared by Brown's extraction and purification procedure. However, the fluorimetric methods were found not to be specific enough for these extracts, the results obtained being erroneously high. Consequently a micro-modification of the Kober reaction was investigated and this proved to give satisfactory results with a high degree of specificity. The second section of this thesis therefore describes studies on the fluorescence reactions of pure oestrogens and the failure to apply

them to blood extracts; this is followed by the development of a 'micro'-Kober reaction for the determination of 0.05 - 1 µg. oestrogen. Using the 'micro'-Kober reaction, it was possible to adapt the method of Brown (1955), which measures urinary oestrogens, for the determination of oestrogens in blood from pregnant women, and in foetal umbilical cord blood at delivery. The assay method which was finally developed is described in detail and its reliability, i.e. its accuracy, specificity and sensitivity, are discussed.

The third section of this thesis is concerned with the application of this method to studies in normal and abnormal pregnancies, to calculations of the ratio of blood concentration to urine concentration in normal pregnancy and to comparisons between foetal and maternal levels at Caesarean section.

II. DEVELOPMENT OF THE METHOD

II. DEVELOPMENT OF THE METHOD

The reagents and apparatus used throughout this section are described in the Appendix.

A. Methods of Detection of Oestrogens

1. Fluorimetry

Both phosphoric and sulphuric acids can be used for producing a fluorescence with the oestrogens. Finkelstein, Hestrin & Koch (1947) described a method using phosphoric acid. Solutions of oestrogens in ethanol were evaporated first in a water-bath and then in an oven at 110° for 1 hour. Three millilitres of phosphoric acid were added, the tubes were stoppered and heated in a water-bath at 95° in the dark for 30 minutes, and cooled. The fluorescence faded with increased heating time, if exposed to light or if water entered the tubes during the heating period. These workers used this reaction for measuring up to 5 µg. oestrone, 2.5 µg. oestradiol and 10 µg. oestriol. About the same time, Jailer (1947) developed a quantitative fluorescence reaction using 60-70% H_2SO_4 (v/v). Subsequently several other workers developed quantitative methods for the determination of oestrogens by measurement of the fluorescence with sulphuric acid. The absorption maximum of oestrogens in the H_2SO_4 reaction is about 436 mµ, while the maximum of the fluorescent light is 525 mµ. Either interference filters or glass filters may be used. Interference filters can be used to give increased specificity by virtue of the narrow wavelength band transmitted, but they have a low transmission, and can be used only with higher concentrations.

TABLE 2.

COMPARISON OF FLUORESCENCE METHODS

Reference	Suitable for concentrations of:- oestriol (all in µg.)	oestrone	oestradiol	Reagent used and volume of reagent used
Finkelstein et al. (1947)	3-10	5	2.5	3 ml. phosphoric acid.
Jailer (1947)	0.5-2.5	0.05-0.25	0.05-0.25	8 ml. 60% H_2SO_4 (V/V)
Bates & Cohen (1947, 1950) 1)	5	5	5	1 ml. 90% H_2SO_4 diluted with 6 ml. 65% H_2SO_4 after heating.
Bates & Cohen (1947, 1950) 2)	0.1-0.5	0.1 -0.5	0.1 -0.5	1 ml. 90% H_2SO_4 diluted with 6 ml. 65% H_2SO_4 after heating.
Engel (1950)	0.4	0.3 (in alcohol:toluene) (1:19)	0.4	1 ml. 90% H_2SO_4 diluted with 7 ml. 65% H_2SO_4 after heating.
Diczfalusy (1953)	0.2-1.0	0.2-1.0	0.2-1.0	8 ml. 88% H_2SO_4 (V/V)
Brown (1952)	0.3-2	0.02-0.2	0.02-0.2	0.1 mg. catechol (0.1% EtOH solution) added before evaporation of solvents.

1) oestriol 1 ml. 76% H_2SO_4 (V/V) containing 0.2% (W/V) AS_2O_3.

2) oestrone, oestradiol 1 ml. 68% H_2SO_4 (V/V) containing 0.2% (W/V) AS_2O_3.

TABLE 2.

USED BY VARIOUS WORKERS

Heating period	Filter systems used
30 min. water-bath 98°	
5 min. boiling water-bath	Read at two exciting wavelengths, 436 mμ and 365 mμ.
10 min. 100°	Use of interference filters for specificity or glass filters for sensitivity
10 min. 80°	Interference filter as secondary filter, glass filter as primary filter.
10 min. boiling water-bath	Interference filter as secondary filter, glass filter as primary filter unless greater sensitivity is required when glass filter in primary position and read at 436 mμ - 360 mμ.
	Corning glass filters 5113 and 3384 as primary and secondary filters respectively.
oestriol 20 min. boiling water bath	
oestrone, oestradiol 8 min. boiling water bath	

On the other hand, glass filters transmit very much more light over a wider wavelength band; accordingly they give increased sensitivity but lower specificity. Several methods for measuring oestrogens by the intensity of their fluorescence with sulphuric acid are summarised in Table 2.

Fluorescence of oestrogen methyl ethers

The fluorescence method of Brown (1952) was first studied and a series of calibration graphs were prepared using pure oestrogen methyl ethers. A sample calibration graph is shown in Figure 1. Benzene (6 ml.) was added to each tube before evaporation. There were considerable day to day variations in the intensity of fluorescence given by the same amount of standard, the fluorimeter being set at 100 for the highest concentration of standard used (which was usually 0.1-0.2 µg. of oestrone and oestradiol and 0.4-2.0 µg. oestriol). Occasionally aberrent readings which bore no relation to the concentration of oestrogen present were obtained.

In preliminary experiments using the method it was found that catechol gradually accumulated in the evaporation apparatus and darkened on exposure to air. This then contaminated the oestrogen fractions giving erratic results. Frequent cleaning of the apparatus with chromic acid was therefore necessary. Further investigation showed that the catechol could be omitted with advantage without diminishing the fluorescence of the oestrogens, provided the tubes were stoppered during heating.

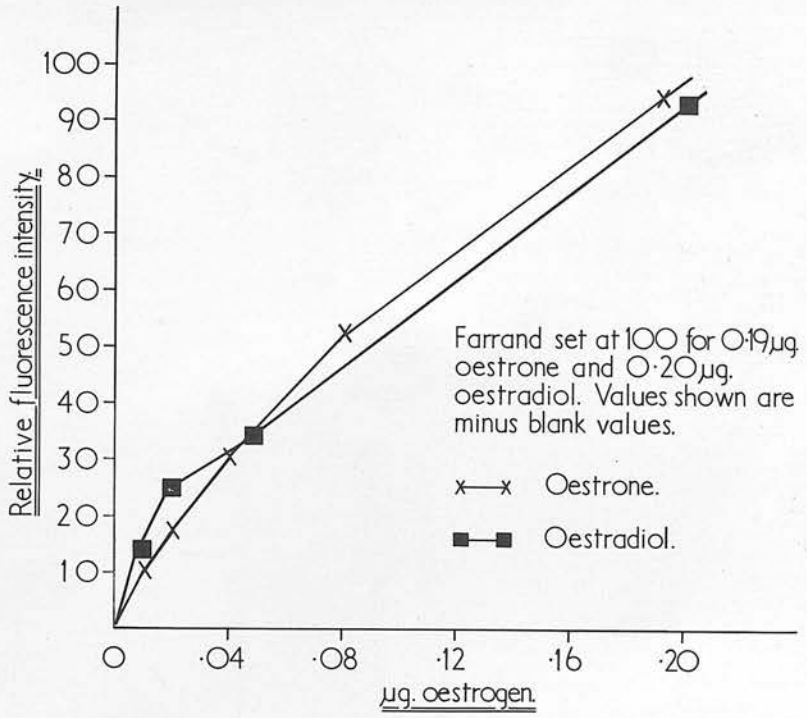


Fig.1a. Relationship between fluorescence intensity and and amount of oestrogen.

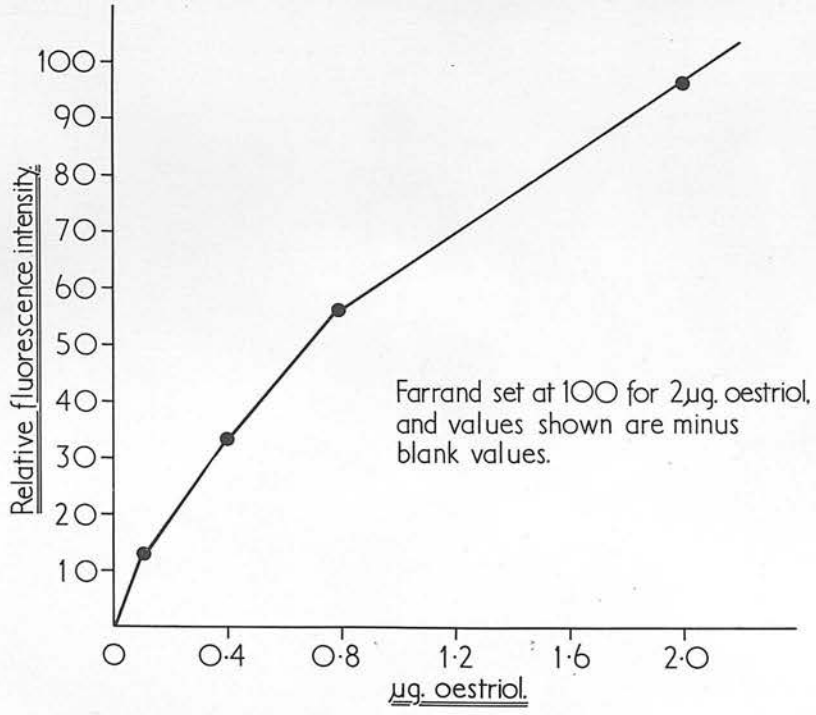


Fig.1b. Relationship between fluorescence intensity and amount of oestriol.

The method finally adopted was as follows:- Light petroleum-benzene (40/60, 7 ml.) was added to oestrone dissolved in 0.1-0.4 ml. ethanol, and to a blank tube; similarly benzene (6 ml.) was added to the oestradiol and oestriol tubes and blank tubes. The solvent was evaporated and 1 ml. of the appropriate sulphuric acid - arsenious acid reagent was added to each tube. The tubes were stoppered and heated in a boiling water-bath for 8 minutes for the oestrone and oestradiol; 20 minutes for oestriol. The tubes were shaken when put in the water-bath and again after 5 minutes when the stoppers were released. Specimen calibration graphs of the relative fluorescent intensity/concentration are shown in Figure 2. The graphs shown are the means of 7 such calibration graphs which had been prepared at approximately monthly intervals to determine whether any changes in linearity occurred.

The intensity of the fluorescence produced by the above procedure was compared with that given by several of the methods listed in Table 2. No benzene or benzene/light petroleum was added to the standards when using these fluorescence procedures and the filters used throughout were Corning glass filters 5113 and 3384 as the primary and secondary filters respectively. The results of these comparisons are shown in Figures 3, 4 and 5. Since the final volume was different for each method, the results have been corrected to a final volume of 1 ml. for comparison. With each of the oestrogens, Engel's method gives the greatest intensity of fluorescence. Diczfalusy's method, the method of Bates & Cohen and the method developed here all give about the same

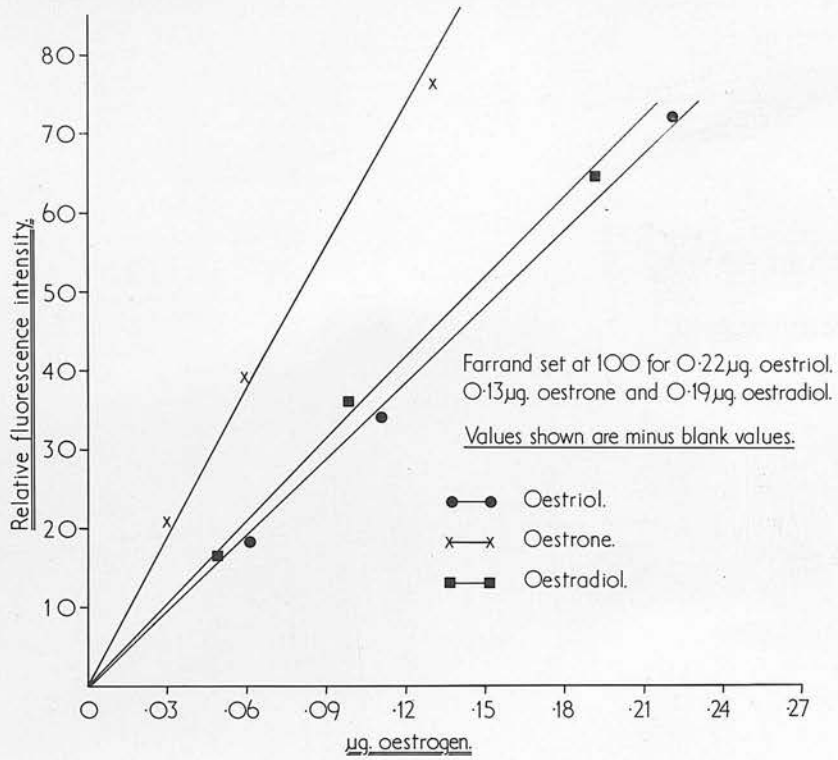


Fig. 2. Relationship between fluorescence intensity and amount of oestrogens.

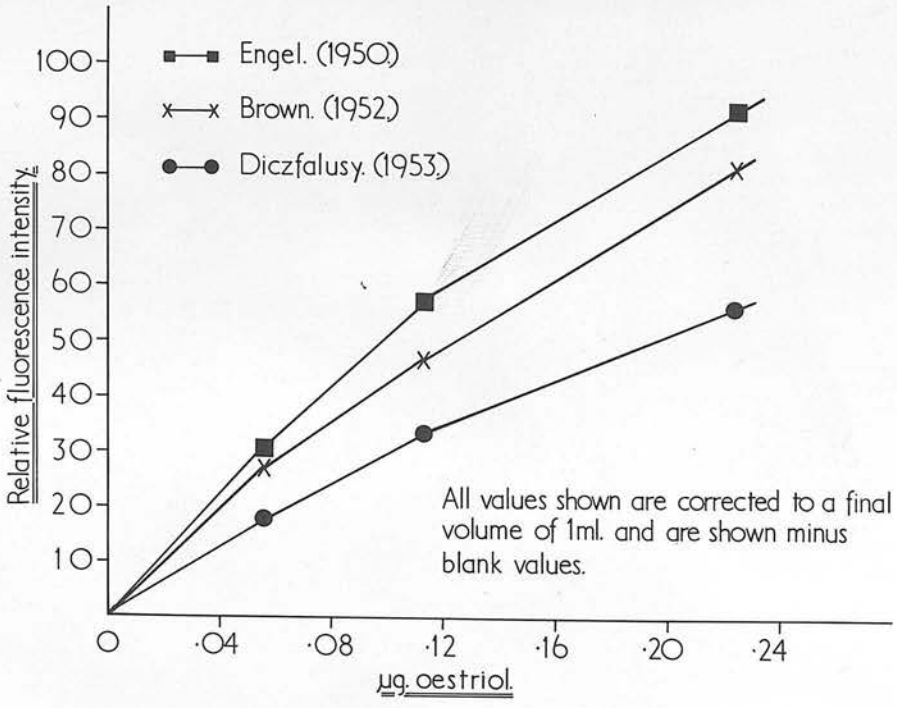


Fig. 3. The relationship between the amount of oestriol and the fluorescence intensity given by different methods.

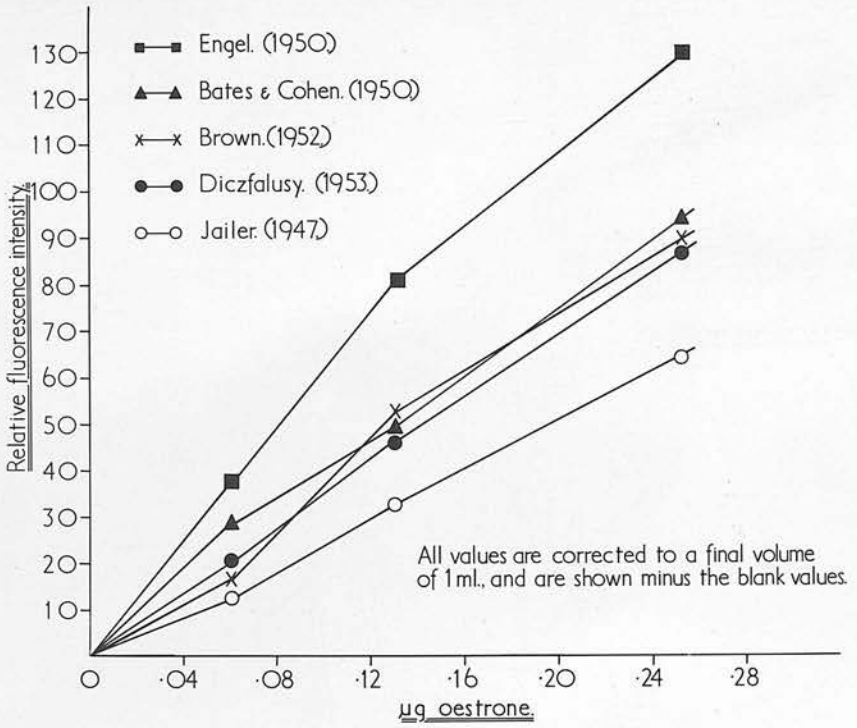


Fig. 4. The relationship between the amount of oestrone and the fluorescence intensity given by different methods.

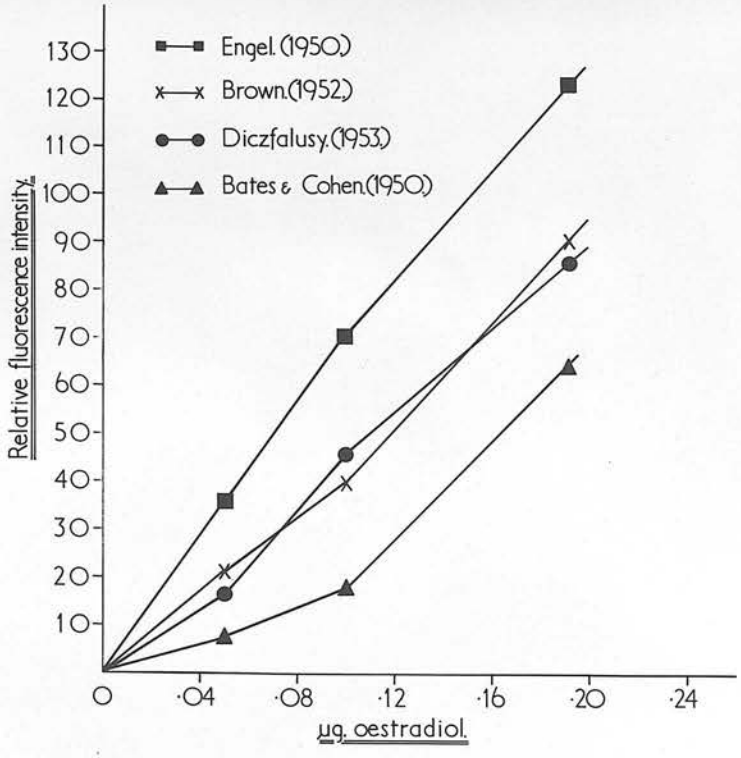


Fig. 5. The relationship between the amount of oestradiol and the fluorescence intensity given by different methods.

intensity for oestrone, while with oestradiol the method of Bates & Cohen gives a much lower florescence intensity than do the other two methods. Diczfalusy's method gives a much lower intensity with oestriol than the method developed here. In these experiments arsenious acid did not have the enhancing effect claimed by Brown. Perhaps the role of the reducing agent was taken by the alcohol present in the other fluorescent reactions.

The method appeared as satisfactory as any for measuring 0.02-0.3 µg. oestriol, oestrone and oestradiol in benzene, benzene-ethanol or benzene-light petroleum solutions and was immediately applicable without modification to the Farrand fluorimeter which was available. It was therefore applied to the measurement of oestriol, oestrone and oestradiol in extracts which had been prepared from cord blood and blood from non-pregnant subjects by a modification of the urinary method of Brown (1955).

Fluorescence of blood extracts

Two millilitres of cord blood or blood from non-pregnant individuals were diluted to 100 ml. with water and hydrolysed by boiling with 20 vol.% concentrated HCl for 60 minutes. After cooling the blood was extracted once with 100 ml. ether and twice with 50 ml. ether, centrifugation being used to separate stubborn emulsions. The ether extracts were washed with concentrated carbonate solution of pH 10.5, evaporated to dryness, methylated and chromatographed as described for urine by Brown (1955). The oestrogen content of the final fractions was determined by the fluorimetric method just described.

Recovery experiments were carried out by adding oestrogens to cord blood and to blood from non-pregnant women, both before and after hydrolysis. The recoveries are given in Table 3. The recovery rates of oestrone and oestradiol were not as high as those for the urinary procedure and were more variable. However they were considered satisfactory for a preliminary study of the concentration of oestrogens in extracts of blood from various sources.

TABLE 3.

Recovery of Oestrogens from Blood

Type of blood used	Oestriol		Oestrone		Oestradiol	
	µg added /10 ml.	% Recovery ± S.D.	µg added /10 ml.	% Recovery ± S.D.	µg added /10 ml.	% Recovery ± S.D.
* Cord blood (8)	1.3	90 ± 10	0.14	65 ± 21	0.13	72 ± 16
* Blood from non-pregnant women (6)	1.3	108 ± 14	0.14	75 ± 12	0.15	81 ± 13
† Blood from non-pregnant women (8)	1.3	76 ± 31	0.14	51 ± 12	0.15	52 ± 16

Figures in parenthesis refer to the number of experiments.

* Oestrogens added to hydrolysed blood.

† Oestrogens added to diluted blood before hydrolysis.

The oestrogen concentrations in pooled cord blood and blood from men and from women having normal menstrual cycles were determined by the above method and the mean results with their standard deviations are summarised in Table 4.

TABLE 4.

Type of blood assayed	Oestriol µg/100 ml.	Oestrone µg/100 ml.	Oestradiol µg/100 ml.
Cord blood (10)	61.6 ± 4.1	3.7 ± 1.4	4.4 ± 3.3
Blood from non-pregnant women (8)	2.8 ± 1.1	3.0 ± 1.2	3.9 ± 1.8
Male blood (8)	4.8 ± 0.44	2.3 ± 0.44	5.8 ± 1.7

At the time these measurements were made, reliable figures for the oestrogen content of cord blood and the blood of non-pregnant individuals were not available. In view of the values found in the urine of pregnant women, where oestriol is the major oestrogen, the value for cord blood seemed reasonable. The marked difference between the oestriol content of foetal and maternal blood was not known at that time. The surprising feature of the results shown in Table 4 was that the oestrone and oestradiol content of blood from non-pregnant individuals was the same as the oestrone and oestradiol content of cord blood. On the basis of the urinary figures, which are some 100-1000 times greater in pregnant women at term than in non-pregnant individuals, less than 0.05 µg. of oestrogen might have been expected to be present in the fractions from non-pregnancy

blood, assuming that the renal clearance in the two states is the same. The high values were not due to solvents used throughout the method, because when 100 ml. water was treated as 2 ml. blood diluted to 100 ml., the 'concentrations' of oestriol, oestrone and oestradiol in the final extracts were equivalent to 0.1, 0.03 and 0.02 µg. per 100 ml. water respectively. The results could have been due to the presence, in the blood extracts, of impurities which also fluoresced when heated with sulphuric acid. If this were true, the fluorescence reaction would not be suitable for the determination of oestrogens in blood extracts prepared by the method of Brown, although it had been satisfactory for the measurement of oestrogens in pure solution. Either greater purification of the extracts or a more specific method of detection was required.

Later Preedy & Aitken (1957) using the fluorescence reaction to measure oestrogens in blood and urine extracts which have been purified by gradient elution chomatography, found that specificity could be obtained only by observing the behaviour of the fluorescent material during chromatography and by measuring the fluorescence of each of the many fractions eluted.

The highly specific Kober reaction supplied the necessary specificity for measuring oestrogens in urine extracts prepared by the method of Brown. However, the Kober reaction as used for urine is not sensitive enough for blood extracts when only 5-10 ml. blood are available. This reaction is sensitive enough for measuring 0.5-10 µg. oestrogen in a final volume of 3-4 ml., which is approximately 10 times the range required for pregnancy blood. The possibility of developing a 'micro'-Kober reaction, using a

final volume of 0.3-0.4 ml. and micro-cuvettes with the same light path of 10 mm. for colour measurement and therefore giving ten times the sensitivity, was investigated. The Kober reaction as described by Brown (1955) for use with urinary extracts will be termed the 'macro'-Kober reaction in this thesis.

2. The 'Micro'-Kober Reaction

When designing the 'micro'-Kober reaction a final volume of 0.3-0.4 ml. was considered to be the minimum that could be handled conveniently without taking very special precautions. Furthermore, glass cells with a light-path of 10 mm. and a capacity of 0.4 ml. were available from Hilger & Watt of London. At that time no carrier was available for positioning these cells in the Unicam SP 600 spectrophotometer, the preferred instrument for making the optical density measurements. Dr. J.B. Brown, of this laboratory, consequently accepted the task of designing and making the necessary cell carrier. The device which resulted accepted 5 glass cells and enabled optical density measurements to be made on four 'micro' samples with the same precision and convenience as the normal 'macro' assembly of the spectrophotometer. The design was subsequently adopted by Unicam of Cambridge and is now available commercially.

The 'micro'-Kober reaction was modified directly from the 'macro'-Kober reaction, the same reagents, water concentrations and heating times being employed, the only difference being that the volumes were reduced to exactly one-tenth. The procedure is fully described later in the section giving complete details of the assay method. Bauld (1954) showed that the size of the tubes (which determines the ratio of surface area to volume of the reaction mixture) has an important effect on the intensity of colour

produced in the Kober reaction. Consequently the tubes for the 'micro'-Kober reaction were designed in such a way that they would hold the complete eluate from the chromatography columns, and yet have a constricted portion where the 'micro'-Kober reaction could be performed with approximately the same ratio of surface area to volume as that used for the 'macro' reaction. The adopted tube was 12 cm. long and 1½ cm. diameter, narrowed at one end to a diameter of 10 mm. for a length of approximately 10 mm., and fitted at the other with a B 14 socket for connection to the evaporation apparatus. These will be referred to as "micro-Kober tubes". The reaction is carried out in the narrowed section of the tube. Care is required when evaporating solvents so that the residue collects in the narrowed section and is not spread over the inner walls of the tubes. This is achieved by immersing only the narrowed section of the tubes in the water-bath until about a ml. of solvent remains; in this way the solvent distils slowly, condenses on the walls of the tubes, and refluxes the residue into the narrowed portion of the tube. The tubes are then lowered in the water-bath and the remainder of the solvent is evaporated and the tubes dried completely.

Reagent blanks are prepared by evaporating 0.05 ml. ethanol containing 0.5 µg. quinol in a 'Kober' tube and adding 0.3 ml. of the appropriate Kober reagent. These are treated in exactly the same manner as the tubes containing pure oestrogens or blood extracts containing oestrogens. The optical densities of the Kober colour are measured in the 0.4-0.5 ml. cuvettes in the micro-cell carrier against the appropriate reagent blanks.

The Kober reagents and the water added before the second heating are measured into the 'micro'-Kober tubes, using 1 ml. graduated pipettes. The Kober reagent is run round the lower walls of the tubes by revolving the tubes in a semi-horizontal position to dissolve the residue and the quinol. This is repeated immediately after the tubes have been put in the boiling water-bath to ensure that all the residue has dissolved, particularly in the case of the oestradiol where it is usually difficult to dissolve all the residue when cold. After developing the colour, the solutions are transferred by a Pasteur pipette to the cuvettes for reading, taking care not to trap any air bubbles. The cuvettes are emptied by a brisk shake, drained on filter paper, then rinsed with 30% H_2SO_4 and drained again, before being reused. The tubes are not specially covered during the heating periods but are carefully protected from contamination with dust if left for any length of time. Interference by dust is much more noticeable with the 'micro'-Kober reaction than with the 'macro'-Kober reaction because of the small volumes used (see page 53).

The optical densities (D) of the oestriol and oestrone methyl ethers are read at 480, 516 and 552 mμ, and of the oestradiol methyl ether at 480, 518 and 556 mμ; 516 mμ being the absorption maximum of oestriol and oestrone, and 518 mμ. that of oestradiol, 480, 552 and 556 mμ. being wavelengths equidistant from the maximum. The corrected optical densities are given by the following formulae, derived from that of Allen (1950):-

Oestriol and oestrone corrected reading $= 2D_{516}-(D_{480}+D_{552})$

Oestradiol corrected reading $= 2D_{518}-(D_{480}+D_{556})$

TABLE 5

The Relationship between corrected Spectrophotometer Readings and the Amounts of Oestrogen Methyl Ethers

(expressed in terms of the weight of the free oestrogens)

oestrogen (µg)	Corrected Readings (mean ± S.D.)		
	oestriol	oestrone	oestradiol
0.1	0.050 ± 0.009 (14)	0.057 ± 0.009 (14)	0.046 ± 0.011 (13)
0.2	0.099 ± 0.013 (14)	0.127 ± 0.021 (14)	0.118 ± 0.010 (13)
0.4	0.185 ± 0.011 (16)	0.265 ± 0.024 (15)	0.244 ± 0.019 (15)
* 0.4	0.167 ± 0.018 (5)	0.256 ± 0.020 (8)	0.248 ± 0.012 (16)
0.8	0.350 ± 0.023 (15)	0.503 ± 0.046 (12)	0.487 ± 0.026 (12)

All values shown were obtained with added solvent except those marked with an asterisk where the standards were evaporated from 0.4 ml. of ethanol. Figures in parentheses refer to the number of observations.

Allen's original formula was:-

$$\text{corrected reading} = D_{516} - \frac{D_{480} + D_{552}}{2}$$

but double this expression is used here to avoid fractions obtained when dividing by two.

Calibration graphs are prepared at monthly intervals with pure oestrogen methyl ethers and are calibrated in terms of the corresponding amounts of free oestrogens by multiplying by the ratio of the molecular weights (0.95). Volumes of solvents, corresponding to the volumes of eluates containing the oestrogens after chromatography, are added to the standards before evaporation i.e. 8 ml. benzene/light petroleum, 6 ml. benzene and 8 ml. ethanol/benzene to the oestrone, oestradiol and oestriol standards respectively, but not to the blank tubes. This is done to allow for the impurities present in the solvents which give rise to yellow-brown colours in the Kober reaction (see Section II, D, 3).

The mean corrected readings $\pm$S.D. of 12-16 such standard graphs obtained over a period of 2 years are shown in Table 5 and the means are plotted against concentration in Figure 6. The optical densities are almost identical to those obtained with 10 times the amount of oestrogen using the 'macro'-Kober reaction shown in Figure 7.

The formation in the Kober reaction of a pink colour with a maximum at 515 mμ is a highly specific property of the natural oestrogens. However, impurities in the solvents or blood extracts can give rise to yellow colours on heating with Kober reagents. The contaminants arise from several sources. In the case of the 'micro'-Kober reaction, solvents play a large part in contributing to the interfering chromogens,

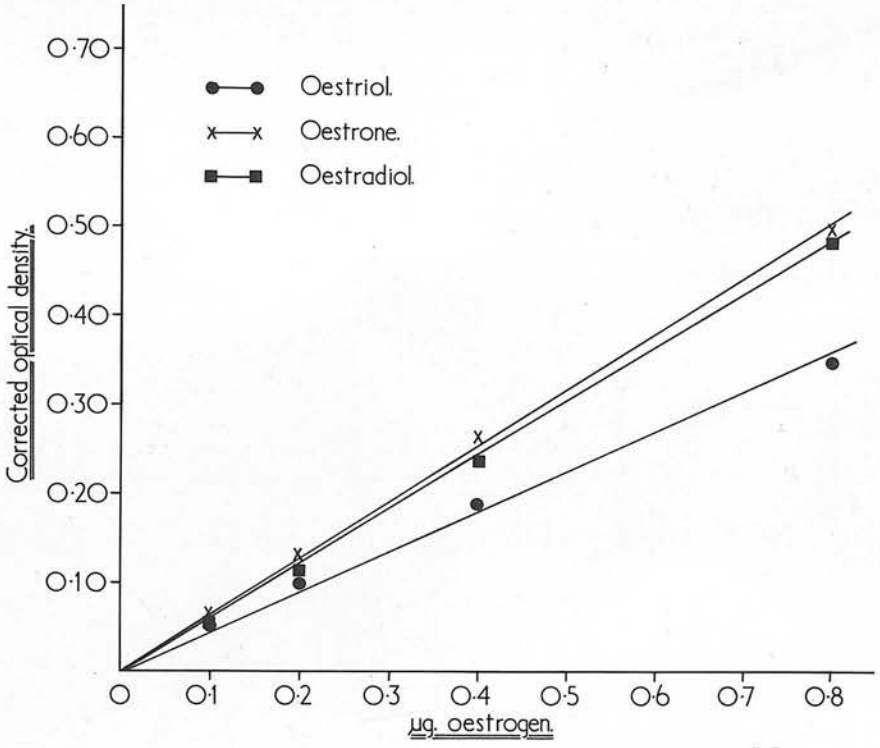


Fig.6. Relationship between corrected spectrophotometer readings and 0·1–0·8 µg. oestrogen methyl ether expressed in terms of the weight of free oestrogen.

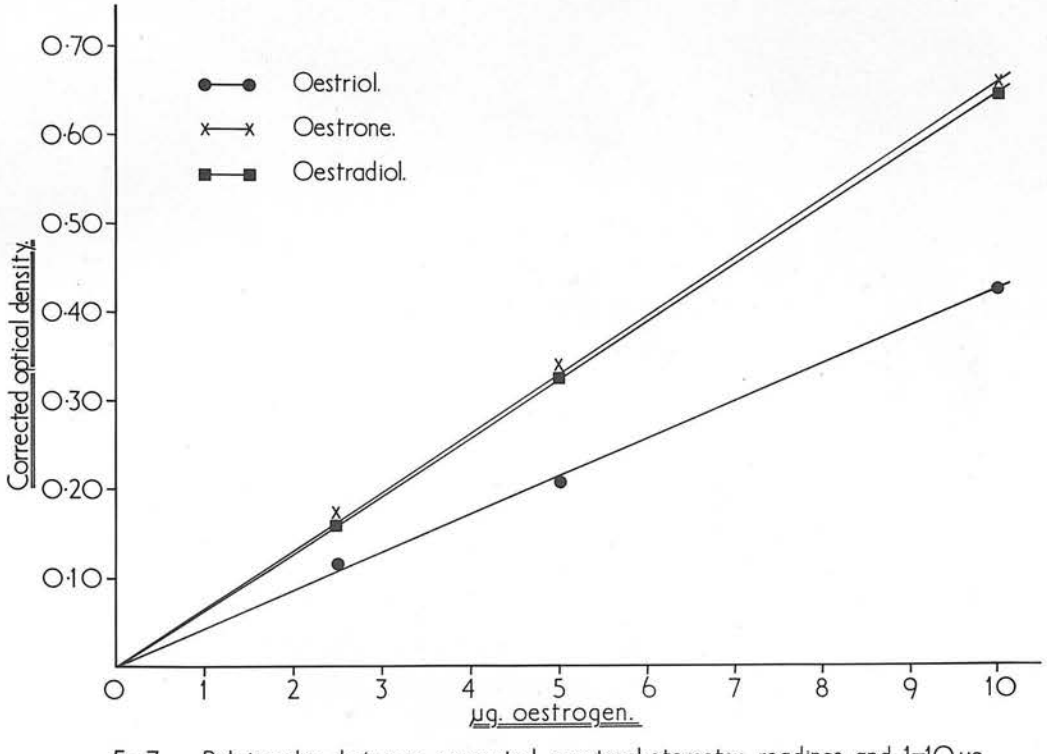


Fig.7. Relationship between corrected spectrophotometer readings and 1–10 µg. oestrogen methyl ether expressed in terms of the weight of free oestrogen.

as does the quinol which is necessary to obtain optimum colour development (Bauld, 1954). The smallest amount of quinol necessary to give the most consistent results was therefore investigated.

In Allen's original formula, substituting the appropriate wavelengths for the oestrogens, the corrected optical density of the oestrogen, i.e. $CDE = OD_{516} - \frac{OD_{480} + OD_{552}}{2}$ (I) (Allen, 1950), represents only a fraction of the total optical density at the absorption maximum. Allen, however, derived a second formula related to equation I :-

$$DE_{516} = \frac{D_{516} - \frac{D_{480} + D_{552}}{2}}{1 - \frac{(k_1 + k_2)}{2}} \quad \text{(II)},$$

where DE is the actual density at 516 mμ. given by the oestrogen. This gives the total optical density produced by the compound in terms of the observed optical densities at the three wavelengths. This expression involves two constants, k_1 and k_2, which are calculated from the optical densities given by the pure compound, k_1 being $\frac{D_{480}}{D_{516}}$ and k_2 $\frac{D_{552}}{D_{516}}$.

The advantage of I, which is the formula most commonly employed in steroid analysis, is that it involves no constants, and can be used therefore even when pure standards are not available. The second expression can be used to calculate the true optical density given at the absorption maximum by oestrogens in the presence of impurities, if k_1 and k_2 are known. Equation II can be rearranged as follows:-

$$DE_{516} = \frac{D_{516} - \frac{D_{480} + D_{552}}{2}}{1 - \frac{(k_1 + k_2)}{2}}$$

$$\frac{DE_{516}}{CDE} = \frac{1}{1 - \frac{(k_1 + k_2)}{2}} \quad \text{(III)}$$

The expression $\frac{1}{1 - \frac{(k_1 + k_2)}{2}}$ can be readily calculated by substitution of the values for k_1 and k_2 or from the ratio of the optical density given by the oestrogen at the absorption maximum to the corrected optical density. This latter calculation can be made from the readings given by standards used for calibration graphs. In the case of the formula used in this thesis, where $D_{Corr} = 2CDE$, $\frac{DE_{516}}{D_{Corr}} = \frac{1}{2 - (k_1 + k_2)}$.

An example of this applied to standards is shown in Table 6 where the expression $\frac{1}{2 - (k_1 + k_2)}$ $\left(\text{or } \frac{DE_{516}}{D_{Corr}}\right)$ gave a mean value of approximately 0.75. This factor was then used to calculate the true optical density given by the oestrogens in the presence of impurities and hence, by subtraction from the observed optical density, the optical density of the impurities themselves. This reduces to the following formula:-

$$D_{Max} = DI_{Max} + DE_{Max}$$

$$DI_{Max} = D_{Max} - DE_{Max}$$

$$= D_{Max} - D_{Corr} \times 0.75. \quad \text{(IV)}.$$

where DI_{Max} is the optical density of the impurities at the absorption maximum; other symbols as explained previously.

When the 'micro'-Kober reaction was carried out with standards, the ratio varied from 0.85 to 1.88 (see Table 7). This difference from the ratio obtained in the 'macro'-Kober reaction is

TABLE 6.

Relationship D_{max} to D_{corr} for Pure Oestrogen Methyl Ethers in the 'macro'-Kober Reaction.

OESTROGEN	D_{480}	D_{max}	D_{552}	D_{corr}	$\frac{D_{max}}{D_{corr}}$.
Oestriol	0.085	0.154	0.018	0.205	0.75
	0.171	0.320	0.049	0.420	0.76
	0.265	0.482	0.075	0.624	0.77
	0.080	0.150	0.022	0.198	0.76
	0.269	0.482	0.072	0.623	0.78
Oestrone	0.060	0.126	0.013	0.179	0.71
	0.130	0.260	0.039	0.351	0.74
	0.271	0.530	0.085	0.708	0.75
	0.068	0.135	0.024	0.178	0.76
	0.142	0.275	0.044	0.364	0.75
	0.300	0.559	0.107	0.711	0.79
Oestradiol	0.065	0.124	† 0.016	0.167	0.74
	0.150	0.252	0.031	0.323	0.78
	0.140	0.250	0.029	0.331	0.75
	0.230	0.478	0.051	0.675	0.71
	0.143	0.250	0.032	0.325	0.79
	0.266	0.495	0.043	0.661	0.75

† D_{556}.

TABLE 7

Relationship D_{Max} to D_{Corr} for pure Oestrogen Methyl Ethers in the 'micro'-Kober Reaction.

OESTROGEN	D_{480}	D_{Max}	D_{552}	D_{Corr}	$\frac{D_{Max}}{D_{Corr.}}$
Oestriol	0.152	0.155	0.053	0.105	1.47
	0.186	0.220	0.059	0.195	1.12
	0.279	0.375	0.101	0.370	1.01
	0.176	0.164	0.065	0.087	1.88
	0.172	0.209	0.081	0.165	1.27
	0.300	0.371	0.130	0.312	1.19
	0.149	0.192	0.046	0.189	1.01
	0.275	0.370	0.107	0.358	1.04
Oestrone	0.140	0.160	0.049	0.131	1.23
	0.190	0.264	0.075	0.263	1.01
	0.385	0.511	0.201	0.436	1.17
	0.140	0.215	0.073	0.217	0.99
	0.329	0.446	0.209	0.354	1.25
	0.137	0.136	0.045	0.090	1.51
	0.286	0.434	0.109	0.473	0.92
	0.225	0.276	0.096	0.231	1.19
Oestradiol	0.058	0.059	0.020	0.040	1.48
	0.088	0.107	0.027	0.099	1.08
	0.165	0.231	0.066	0.231	1.00
	0.257	0.391	0.062	0.463	0.85
	0.105	0.129	0.037	0.116	1.11
	0.176	0.235	0.052	0.242	0.97
	0.304	0.430	0.091	0.465	0.93
	0.190	0.251	0.068	0.244	1.03

† $\frac{D_{556}}{}$

TABLE 8

Effect of Quinol on the Amount of Impurity present in the Kober Colours.

Concentration of quinol	Oestriol µg.	Optical densities. 480 mµ.	516 mµ.	552 mµ.	Corrected optical density		Calculated optical densities of impurities.
0.25 mg.	0.1	0.202	0.165	0.078	0.050		0.127
"	0.2	0.084	0.090	0.026	0.070		0.037
"	0.4	0.100	0.138	0.029	0.147		0.028
"	0.8	0.225	0.303	0.075	0.300		0.073
						Mean	0.066
0.5 mg.	0.1	0.048	0.051	0.014	0.040		0.021
"	0.2	0.074	0.090	0.025	0.081		0.029
"	0.4	0.105	0.148	0.031	0.160		0.028
"	0.8	0.144	0.235	0.036	0.290		0.017
						Mean	0.024
1 mg.	0.1	0.180	0.155	0.075	0.055		0.114
"	0.2	0.160	0.153	0.061	0.085		0.089
"	0.4	0.195	0.216	0.076	0.161		0.095
"	0.8	0.292	0.354	0.079	0.337		0.102
						Mean	0.100
2 mg.	0.1	0.135	0.110	0.048	0.037		0.079
"	0.2	0.265	0.221	0.079	0.098		0.147
"	0.4	0.185	0.200	0.068	0.147		0.090
"	0.8	0.270	0.311	0.084	0.268		0.110
						Mean	0.107

due to the effect of impurities from solvents etc. which are diluted out in the 'macro'-Kober reaction, but which become appreciable in the small volumes used in the 'micro'-Kober reaction.

Using formula IV the optical densities of the impurities present in a series of standards plus solvents, to which various amounts of quinol had been added were calculated. The results which are summarised in Table 8 show that 0.5 mg. quinol gives the most consistent readings with the least amount of colour from impurities. This amount is one-eighth that used in the 'macro'-Kober reaction.

The presence of impurities both in standards and blood extracts will be fully discussed when dealing with the specificity of the method.

B. Extraction and Purification Procedures.

1. Processing the blood.

Several methods for extracting oestrogens from blood were investigated. From the nature of blood oestrogen complexes (Rakoff, Paschkiss and Cantarrow, 1943; Szego and Roberts,1946; Preedy and Aitken, 1957), some form of hydrolysis is required before the conjugated and protein-bound oestrogens can be extracted from blood. Hydrolysis of oestrogen conjugates can be performed by boiling with acid or by incubation with enzyme preparations containing β-glucuronidase and phenolsulphatase. However, these enzymes do not affect protein-bound oestrogens, while acid treatment has the advantage of disrupting the protein-oestrogen link as well as hydrolysing the oestrogen conjugates. Accordingly, acid hydrolysis was investigated as a means of liberating oestrogens directly from blood or from blood extracts obtained by prior treatment with mixtures of alcohol and ether.

10 ml. cord blood were added slowly to 6 volumes ethanol-ether (3 : 1). After centrifuging, the supernatant was evaporated almost to dryness and the residue was dissolved in 200 ml. water. The precipitate was similarly added to 200 ml. water. Both fractions were hydrolysed by boiling with 10 vol.% concentrated HCl for 60 minutes. After cooling, the hydrolysed fractions were extracted with 200 ml. ether and twice with 100 ml. ether. The ether extracts were processed by the method which is described later. After chromatography, the eluates were evaporated to dryness and the oestrogen content determined by the 'micro'-Kober reaction. While

much of the oestrogen was found in the alcohol-ether fraction, a considerable proportion was found in the protein precipitate.

Blood was also diluted to 200 ml. with water, hydrolysed directly by boiling with 10 vol.% concentrated HCl and extracted and processed as above. When the results were compared with those obtained by fractionation of the blood with ethanol-ether, it was found that acid hydrolysis of the whole blood yielded as much oestrogen as the protein and supernatant fractions combined. Ethanol-ether extraction, which is more laborious, was therefore rejected in favour of the direct acid hydrolysis of whole blood.

When the method had been further developed, the problem was re-investigated. The results, which are described in Section II,E,2 of this thesis, merely confirmed that acid hydrolysis of diluted blood gives the highest yield of oestrogens.

2. Extraction and Purification Procedure.

The extraction and purification procedure finally adopted was adapted from Brown's method (1955) for the determination of urinary oestrogens.

As shown in the preceding section, acid hydrolysis of diluted blood is the most satisfactory treatment prior to extraction. Consequently, boiling with 15 vol.% concentrated HCl for 1 hour, as for urine, was used. Extraction of the hydrolysed blood with ether leads to the formation of emulsions, a complication which rarely happens when extracting hydrolysed urine. Emulsions also form when oestrone and oestradiol are extracted from benzene/light petroleum with 1.6% NaOH. Care had to be taken when extracting at

these stages.

The final extracts after chromatography contain considerable amounts of Kober chromogenic impurities which are derived from the solvents themselves. These impurities were minimized by reducing the size of the alumina columns so that smaller volumes of eluates were required for eluting the oestrogen methyl ethers. The oestrogens in the final extracts were measured by the 'micro'-Kober reaction, except the oestriol fraction from umbilical cord blood or amniotic fluid, where sufficient amounts are present for them to be measured by the 'macro'-Kober reaction.

(a) The Handling of Emulsions.

Emulsions form during the extraction with ether of blood, plasma or blood protein fractions which have been hydrolysed by boiling with HCl. These are allowed to stand in the separating funnels for 10-15 minutes, when they partially separate; as much of the aqueous layer as possible is then removed and the layer of emulsion is centrifuged at 850 g. r.p.m. for 15-20 minutes, when it separates completely. The dark brown precipitate is discarded. Usually centrifugation is unnecessary after the second and third extractions with ether. At these stages, the aqueous layer is shaken vigorously two or three times when the emulsion usually separates completely and the ether can be decanted off, leaving behind a few millilitres of highly pigmented material.

When the benzene/light petroleum solution is extracted with 1.6% NaOH to remove the oestrone and oestradiol, emulsions form even when the funnels are shaken very gently. These emulsions usually

clear if left to settle for 10-15 minutes, and the contents of the funnels are then swirled, but not shaken. An interfacial solid forms at this stage but this is discarded, since recovery experiments have shown that it contains no oestrogen.

The formation of troublesome emulsions when extracting hydrolysed blood with ether can be prevented by the addition of Bradasol (β-phenoxy ethyl dimethyl dodecyl ammonium bromide) (Preedy, personal communication). However, the Bradasol is also extracted by the ether and interferes later in the method at the stage where oestriol is extracted from the benzene and petroleum mixture with water. The Bradasol causes the water to cling to the walls of the separating funnel and prevents complete separation of the two phases. This difficulty which occurs to a lesser extent when Bradasol is not added, does not happen during the extraction of oestrone and oestradiol with NaOH. The addition of Bradasol did not improve the recovery of oestrogens added to hydrolysed blood and consequently its use was abandoned.

It was also possible that the substances which cause the emulsions and the formation of interfacial solids during the partition of blood extracts between Benzene/light petroleum and 1.6% NaOH might lower the recovery of oestrogens by altering the partition coefficients. Increasing the percentage of light petroleum used from 50% to 70% to counteract any increase in solubility of the oestrogens in the organic phase caused by lipids did not materially alter the extent of the emulsions or the recovery figures. Potassium soaps are more soluble than sodium soaps and

therefore it was thought that the interfacial solid, which might contain some oestrogen, would not form with KOH. Substitution of KOH for NaOH at this stage did not improve the recovery figures; in fact the emulsions which formed tended to be more difficult to separate.

Plasma did not produce any less emulsion at either of these stages. Plasma containing more than the usual amount of lipid material (by visual examination) produced more emulsions than plasma with a lower lipid content, but the recovery of added oestrogen was unaltered.

The protein content of blood or plasma seemed to contribute largely to the formation of emulsions during ether extraction, while the lipids were the major factor in producing emulsions formed during partition between benzene/light petroleum and NaOH.

From these experiments it was concluded that there was no way of preventing emulsion formation at all stages of the method and while the emulsions hindered the rapid processing of blood, they could be handled without too much loss of time.

The results of these preliminary experiments are summarized in Table 9.

TABLE 9

Recovery of Oestrogens added to Blood after Hydrolysis.

Treatment applied to extracts.	OESTRIOL µg added per 10ml	% Recov -ery.	OESTRONE µg added per 10ml	% Recov -ery.	OESTRADIOL µg added per 10ml	% Recov -ery.
Addition of Bradasol to hydrolysed blood	3.0	86	0.2	68	0.2	73
Addition of 24g. NaOH and 30 min. hydrolysis after acid hydrolysis.	3.0	88	0.2	62	0.2	71
35 ml. light petroleum/15 ml. benzene in place of 25 ml. light petroleum/25 ml. benzene.	3.0	77	0.4	73	0.4	66
Use of 1.6% KOH instead of 1.6%.	3.0	79	0.4	57	0.4	61
Specimen of lipaemic plasma.	1.0	74	0.4	58	0.4	70
Oestrogens added prior to extraction with 1.6% NaOH.	3.0	82	0.4	73	0.4	69
Oestrogens added to methylation flasks	3.0	86	0.4	86	0.4	76
Recovery using standard procedure	3.0	74 (60-90)	0.4	73 (66-76)	0.4	72 (63-82)

The formation of emulsions during ether extraction of blood depended on the previous treatment of the blood. When an ether-ethanol extract of blood was evaporated to dryness and the residue dissolved in water, no emulsions formed during subsequent extraction with ether, whether the residue had been hydrolysed or not. Diluted, unhydrolysed blood could be readily extracted with ether without the formation of emulsions. The extent of the emulsions formed during the ether extraction of acid hydrolysed blood depended on the acid concentration used and on the original dilution of the blood. The more dilute the blood, the less emulsion formed; similarly, increasing the acid concentration used for hydrolysis decreased the amount of emulsion formed during the subsequent ether extractions.

(b) Concentration of HCl used for hydrolysis.

When only 2-5 vol.% concentrated HCl were used for the hydrolysis it was extremely difficult to distinguish the interface between the two layers owing to the even distribution of pigment and the density of the emulsion. Also the emulsions required to be centrifuged for a very much longer period of time. From preliminary observations it appeared that the acid concentration used for hydrolysis was not as critical as for the hydrolysis of urine. Similar yields of oestrogens were obtained when hydrolysis was carried out with acid concentrations ranging from 2.5-20 vol. % concentrated HCl. The recovery of oestrogens added to hydrolysed blood also appeared to be independent of the acid concentration used for hydrolysis. Accordingly, hydrolysis with 15 vol.% concentrated HCl (as for urine) was chosen as a satisfactory

hydrolytic procedure. The effect of acid concentration on the yields of oestrogens will be discussed fully in Section II, E, 2.

(c) Chromatography.

The alumina used for the Chromatography was standardised, deactivated alumina prepared exactly as described by Brown (1955). When it was realised that impurities in the solvents affected the wavelength absorption curves of the oestrogen methyl ethers, the volumes of eluates were decreased by reducing the size of the columns. Consequently chromatography was performed in 10 mm. sintered glass filter tubes, porosity no. 3, 5 cm. long and sealed on to a reservoir of 25 ml. capacity. The weight of alumina was reduced to 0.7 g. and the volumes of the eluates then required were approximately half those used with 2 g. alumina. The elution characteristics of pure oestrogen methyl ethers and blood extracts on these alumina columns are shown in Table 10.

(d) Application of additional purification procedures.

Two procedures which have been effective for removing contaminants from urinary fractions without loss of oestrogens have been applied to fractions from blood.

(i) Saponification.

The saponification step devised by B auld (1956) and modified by Brown, Bulbrook and Greenwood (1957)[b] was incorporated in the method and applied to the determination of oestrone and oestradiol in umbilical cord blood. The results of seven comparisons, using blood containing 0.6 - 2.1 µg. oestrone and 0-0.6µg. oestradiol per 100 ml. are summarised in Table 11. The mean differences between the two series of results (results with

TABLE 10

The Elution Pattern of Oestrogen Methyl Ethers and Blood Extracts from Alumina* Columns.

Eluate	BENZENE-LIGHT PETROLEUM.						BENZENE		
	25/75 (v/v)			40/60 (v/v)					
mls of eluate	4	2	4	6	8	10	2	4	6
oestrone methyl ether	-	24	43	31	2	-	-	-	-
oestradiol-17β methyl ether.	-	-	-	-	-	-	24	62	14
blood extract	-	29	42	21	8	-	38	54	8

Eluate	ETHANOL-BENZENE				
	1.4/98.6 (v/v)	2.5/97.5 (v/v)			
mls of eluate	3	3	4	6	8
oestriol methyl ether	-	6	64	28	2
blood extract	-	2	91	5	2

Results are expressed as the percentage of oestrogen present in each fraction, and in the case of blood extracts refer only to the Kober chromogens estimated as oestrogens after applying the colour correction.

* activity as specified, B rown (1955).

saponification minus results without saponification) with their standard deviations were -0.02 ± 0.09 µg/100 ml. for oestrone and -0.04 ± 0.07µg/100 ml. for oestradiol. In addition, the amounts of impurities present, expressed as optical densities at 516 - 518 mµ., were calculated as described in Section II,A,2, and are shown in Table 11. The impurities found in extracts from 10 ml. blood were for the oestrone fraction, means±standard deviations of optical densities (with and without saponification) 0.051±0.023 and 0.061±0.022, and for the oestradiol fraction, 0.051±0.027 and 0.077±0.022. The difference between the two series of results were not greater than that expected from duplicates obtained by the one method. The additional step of saponification was therefore unnecessary in the method for the determination of oestrogens in blood.

(ii) Purification of the oestrone fraction by formation of the Girard complex.

A tenfold purification of urinary oestrone fractions prepared by the method of Brown (1955) has been achieved through formation of the complex formed with Girard's Reagent T (Brown and Blair, 1960). This purification step has been applied to oestrone fractions from umbilical cord blood. The results of eight comparisons using bloods containing 0.9-1.2µg. oestrone per 100 ml. cord blood and which were processed with and without the Girard reaction are summarised in Table 12.

TABLE 11

Effect of Saponification on Oestrone and Oestradiol Fractions from Cord Blood

OESTRONE				OESTRADIOL			
Saponified		Not Saponified		Saponified		Not Saponified	
Dcorr/ 10 ml.	Impurities D_{516}/10 ml.	Dcorr/ 10 ml.	Impurities D_{516}/10 ml.	Dcorr/ 10 ml.	Impurities D_{518}/10 ml.	Dcorr/ 10 ml.	Impurities D_{518}/10 ml.
0.040	0.089	0.041	0.089	-0.005	0.047	-0.003	0.054
0.041	0.032	0.044	0.062	-0.009	0.014	-0.002	0.059
0.134	0.074	0.122	0.087	0.038	0.070	0.032	0.064
0.067	0.033	0.066	0.038	0.008	0.095	0.018	0.075
0.063	0.027	0.064	0.035	0.010	0.048	0.011	0.078
0.060	0.053	0.075	0.065	0.021	0.058	0.029	0.085
0.059	0.051	0.062	0.051	0.025	0.027	0.020	0.121
MEAN* 0.0663	† 0.051 ±0.023	*0.0677	† 0.061 ±0.022	* 0.0123	† 0.051 ±0.27	* 0.015	† 0.077 ±0.022
MEAN as µg/100 ml. 1.11		1.13		0.21		0.25	

* as Dcorr/10 ml.

† as D_{516}/10 ml.

* as Dcorr/10 ml.

† as D_{518}/10 ml.

TABLE 12.

Purification of Oestrone Fraction by Formation of the Girard Complex.

Girard Reaction applied µg/100 ml. Impurities D_{516} /10 ml.		Girard Reaction not applied µg/100 ml. Impurities D_{516} /10ml.	
1.00	0.071	1.00	0.076
0.90	0.054	1.00	0.074
1.10	0.076	1.00	0.075
1.00	0.047	0.90	0.073
1.00	0.074	1.20	0.140
1.00	0.059	1.10	0.082
0.90	0.051	1.00	0.083
1.00	0.204	1.00	0.051
mean 1.00 ±S.D. ±0.08	0.080±0.051	1.02 ±0.09	0.082±0.025

The mean difference between the two sets of results with the standard deviation was -0.01 ± 0.08 µg/100 ml; the calculated optical densities of the impurities at 516 mµ, expressed as the mean±standard deviation, were 0.080 ± 0.051 and 0.082 ± 0.025. There was therefore no difference between the two sets of results. No further purification of the oestrone fraction was therefore achieved by the addition of this step involving the formation of the Girard complex.

(e) Storage of blood for assay.

Some steroids, for example cortisol (Bush, 1957), are metabolised in blood even when kept at 0° C. In this instance plasma must be separated from the red cells shortly after withdrawal.

Werthesen, Baker and Field (1950) found 40-60% loss of oestrone during incubation with whole blood, but this could not be confirmed later (Veldhuis, 1953). Bischoff, Katherman, Favati and Gray (1952) found a 4-5% conversion of oestrone to oestradiol on incubation with blood, which was confirmed by Wall and Migeon (1959). Wall and Migeon showed that this change did not occur in plasma on incubation or in whole blood when the incubation time was less than 30 minutes.

As part of the present investigation an experiment was devised to check on the possibility of loss of oestrogens in blood when kept at 0° C. Twenty millilitres of blood from each of three patients in the last trimester of pregnancy were pooled immediately after withdrawal (the three specimens being withdrawn within 10 minutes of each other). Two 10 ml. portions for duplicate analysis were immediately pipetted into 190 ml. water, to which had been added 30 ml. HCl, and were boiled for 60 minutes. Two 10 ml. portions were immediately centrifuged, the plasma was separated off and the red cells were washed with a volume of 0.9% saline equal to the plasma volume. The saline washings were added to the plasma, the volume was made up to 200 ml. with water and the mixture was boiled with 15 vol. % concentrated HCl for 1 hour, the hydrolysis being started within an hour of withdrawing the blood. The remaining 20 ml. portion of blood was kept at 4° C. for 52 hours before being hydrolysed in

a similar manner. On analysis the results were the same irrespective of the method of treatment (Table 13).

TABLE 13.

Effect of Storage on Oestrogen Concentration.

Treatment	Oestrogen concentration (µg/100 ml)					
	oestriol	mean	oestrone	mean	oestradiol	mean
Blood added to 190 ml. water + 30 ml. conc. HCl	3.9, 4.4	4.15	0.60,0.70	0.65	0.60,0.70	0.65
Plasma + saline wash	4.1, 4.4	4.25	0.60,0.60	0.60	0.70,0.80	0.75
Blood kept at 4° C. for 52 hours	3.9, -	3.9	0.70,0.70	0.70	0.60,0.50	0.55

This indicates that no change of oestrogen occurs in whole blood on storage at 4° C. and that plasma together with the saline washings of the red cells contains practically all of the oestrogens present in the blood.

These and other results showed that special precautions, such as separating the plasma or processing the blood immediately after withdrawal, are unnecessary and storage of blood at 4° C. for periods of up to 5 days is justifiable. Blood was collected by venipuncture from the arm vein of pregnant women or from the umbilical cord by gentle squeezing, into bottles containing 50-60 mg. sodium oxalate to prevent clotting. Partial clotting was found to reduce the precision of the assay.

C. The Method in Detail.

The extraction and purification procedures are summarised in Figure 8.

Flow Sheet for the separation of oestriol, oestrone and oestradiol methyl ethers.

Diluted blood hydrolysed with HCl

| extracted with ether

Ether extract

washed with 1) saturated Na_2CO_3 solution pH 10.5

2) NaOH; NaOH wash neutralised to pH10 with saturated $NaHCO_3$ and shaken again with ether extracts.

3) $NaHCO_3$

4) water.

Ether evaporated.

Residue dissolved in ethanol.
Equal vols. benzene and light petroleum added.

| extracted with water.

Benzene-light petroleum

| extracted with NaOH

NaOH extract (oestrone-oestradiol)

H_3BO_3 added

methylated with dimethyl sulphate at 37°

NaOH and H_2O_2 added

| extracted with light petroleum

Light petroleum extract

washed with water

| chromatographed on alumina

oestrone methyl ether

oestradiol methyl ether

Water extract (oestriol)

| H_3BO_3 added and NaOH added

methylated with dimethyl sulphate at 37°.

NaOH and H_2O_2 added

| extracted with benzene

Benzene extract

washed with water

| chromatographed on alumina

oestriol methyl ether.

Hydrolysis and extraction.

Ten millilitres of whole blood are diluted to 200 ml. with water in a 500 ml. flask and heated to boiling under reflux, when 30 ml. concentrated HCl (11 N) are added. After boiling 60 minutes the hydrolysed blood is cooled under running water. The hydrolysed blood is extracted once with 200 ml. ether and twice with 100 ml. ether, taking care to shake gently. The emulsion formed during the first extraction is centrifuged at 850 g. for 20 minutes after the aqueous layer has been run off. After centrifuging, the remainder of the aqueous phase and all the ether layer are decanted separately, and the layer of dark brown material is discarded. The emulsions which form during the second and third ether extractions usually separate if shaken vigorously after removal of the aqueous phase (see page 33). The ether layers are combined and extracted with concentrated carbonate solution of pH 10.5 (80 mls). The aqueous layer is discarded and the ether is shaken with 20 ml. 8% NaOH, which is then brought to pH 10 by the addition of 80 ml. 8% $NaHCO_3$. The aqueous and ether layers are shaken thoroughly again and the aqueous phase is discarded. The ether is washed with 20 ml. 8% $NaHCO_3$ which is discarded, and finally with 10 ml. water, which is discarded. The water is drained off as completely as possible before the ether is evaporated just to dryness on a water-bath. One millilitre ethanol is added to the warm flask to dissolve the residue.

Extraction of the phenolic fraction and methylation.

The residue in ethanol is transferred to a separating funnel with 25 ml. benzene and 25 ml. light petroleum. This is extracted

with two 25 ml. volumes water (the oestriol fraction) and then carefully with two 25 ml. volumes 1.6% NaOH (the oestrone-oestradiol fraction). Even with gentle shaking emulsions are produced with the NaOH but these usually separate on standing for 10-20 minutes. The layer of interfacial material is rejected. The oestriol and oestrone-oestradiol fractions are each collected in 100 ml. stoppered conical flasks containing 0.9 g. boric acid. Four millilitres NaOH (20%) are added to the oestriol fraction and 1 ml. dimethyl sulphate to both fractions. The flasks are shaken vigorously to dissolve the dimethyl sulphate and are incubated at 37°C. for 30 minutes. A further 2 ml. 20% NaOH and 1 ml. dimethyl sulphate are added to each flask and the flasks are again shaken vigorously. The flasks are kept for a further 30 minutes at 37° C. or overnight at room temperature.

Extraction of the methylated oestrogens.

Two and a half millilitres hydrogen peroxide (30%) and 10 ml. 20% NaOH are added to each flask and the contents are transferred to separating funnels. The methylated oestriol fraction is extracted with 25 ml. benzene and the methylated oestrone-oestradiol fraction with 25 ml. light petroleum, the solvent first being used to rinse out the appropriate methylation flask. The extracts are washed with two 5 ml. volumes of water and the water is drained off as completely as possible.

Chromatography.

The Methylated oestriol fraction.

An alumina column is prepared by pouring 0.7 g. standardised deactivated alumina into a chromatogram tube partly filled with

benzene. After the alumina has settled a 5 mm. layer of sand is added to protect the column when adding solvents. The benzene extract containing the oestriol methyl ether is applied to the column, taking care not to transfer any droplets of water with it. When all the benzene has passed through, the column is eluted with 3 ml. 1.4% ethanol in benzene, the eluate being discarded. The column is eluted with 7 ml. 2.5% ethanol in benzene, which elutes the oestriol methyl ether. This eluate is collected in a tube for evaporation of the solvents and development of the Kober colour.

The methylated oestrone-oestradiol fraction.

The oestrone-oestradiol methyl ether fraction is similarly applied to a second column of alumina (0.7 g.) prepared with light petroleum. This column is eluted first with 4 ml. 25% benzene in light petroleum, which is discarded. It is then eluted with 10 ml. 40% benzene in light petroleum; the first 7 ml. of this eluate contains the oestrone methyl ether and is collected in a tube, the last 3 ml. is discarded. Finally, the column is eluted with 5 ml. benzene to elute the oestradiol methyl ether, which is also collected in a Kober tube.

Evaporation of solvents.

Quinol (0.5 mg) in ethanolic solution (1% w/v) and a small piece of clean porous tile are added to each eluate and to appropriate blank tubes. The tubes are evaporated to dryness under an atmosphere of nitrogen, the rate being controlled by applying a partial vacuum from a water-pump. The last few millilitres are allowed to distil slowly so that the residue

collects in the narrow end of the tube. The tubes are then allowed to stand for a few seconds in the boiling water-bath under full vacuum from the pump to remove all traces of solvents. The tubes are then cooled under a flow of nitrogen.

Development of the Kober colour and colorimetry.

After evaporation, 0.3 ml. of the appropriate Kober reagent is added to each tube, i.e. 2% quinol (w/v) in 76% (v/v) H_2SO_4 for oestriol, 2% quinol in 66% H_2SO_4 for oestrone and 2% quinol in 60% H_2SO_4 for oestradiol. The reagent is allowed to run over the lower walls of the tubes in order to dissolve the residue. The tubes are heated for 20 minutes in a boiling water-bath, the contents being thoroughly mixed after 5 minutes heating. They are then cooled for approximately 10 minutes in cold water, and 0.1 ml. water is added to the oestriol tubes, 0.05 ml. to the oestrone tubes and 0.02 ml. to the oestradiol tubes. The contents are mixed and the tubes are heated a further 10 minutes in the boiling water-bath. They are again cooled in cold water for approximately 10 minutes.

Optical densities are measured against the similarly treated reagent blanks in the spectrophotometer at the following wavelengths: 480, 516 and 552 mμ for oestriol and oestrone methyl ether fractions, and 480, 518 and 552 mμ for oestradiol methyl ether fraction. Optical density readings (D) are corrected by applying the formulae quoted earlier, namely:-

oestriol and oestrone corrected readings = $2\,D_{516} - (D_{480}+D_{552})$

oestradiol corrected reading = $2\,D_{518} - (D_{480}+D_{556})$

The amount of oestrogen present in each tube is calculated from the corrected optical density and the appropriate calibration graph (see Figure 6).

D. Reliability of the Method.

Before a chemical method for the determination of oestrogen is used for clinical studies, the reliability of the method should be known. The accuracy, specificity and sensitivity of the method just described were determined for both cord blood and blood from pregnant women. To obtain levels in recovery experiments of the order of those found in the first trimester of pregnancy, very small amounts of oestrogens were added to male blood.

1. Accuracy.

Recovery experiments were performed to test the accuracy of the extraction, purification and colorimetric stages of the method and to determine whether losses occurred during acid hydrolysis. Pooled foetal blood was obtained from the umbilical cord at delivery. This would be principally umbilical venous blood. Samples of blood were obtained from non-pregnant subjects and women in the last trimester of pregnancy. Amounts of oestrogens corresponding to the levels found from the 12th to 40th week of pregnancy were added to blood, either before or after hydrolysis, and the percentage recovered was determined after subtracting the blank values. The results are summarised in Table 14.

When the oestrogens were added after hydrolysis the mean recoveries were between 54 and 78%. When added before hydrolysis, in the case of cord blood and blood from pregnant women, the recoveries were lower by 3-20%. These recoveries are generally lower than those obtained when the method is applied to urine (Brown, Bulbrook and Greenwood, 1957b). Unsuccessful attempts

TABLE 14 - RECOVERY of OESTROGENS added to BLOOD BEFORE AND AFTER HYDROLYSIS

(All figures corrected for endogenous values)

Source of Blood	Stage when oestrogens were added	Oestriol			Oestrone			Oestradiol		
		µg added per 10ml	No. of Obs.	% Recovery M ± S.D.	µg added per 10ml	No.of Obs.	% Recovery M ± S.D.	µg added per 10ml	No.of Obs.	% Recovery M ± S.D.
Non-pregnant subjects	After hydrolysis	0.05	7	54±20	0.05	10	78±13	0.05	10	69±15
Pregnant women	After hydrolysis	1.0	13	63±8	0.4	12	66±13	0.2	12	67±13
	Before hydrolysis	1.0	6	56±4	0.4	7	56±5	0.2	8	47±5
Foetal cord	After hydrolysis	16	13	64±7	0.4	17	68±9	0.2	23	72±11
	Before hydrolysis	16	11	59±7	0.4	14	65±12	0.2	16	64±14

to improve these figures have already been described. The losses appear to be due to partial absorption on to the hydrolysed protein, to interference in partition coefficients by blood lipids and to the mechanical difficulties caused by the emulsions and interfacial solids encountered during the procedure. These results can be regarded as satisfactory under the circumstances, although higher recovery values and lower standard deviations would be desirable.

2. Precision.

An estimate of the precision of a method is given by the standard deviation (S.D.) of results of replicate determinations from their means. This was calculated by the method of Snedecor (1952) from a series of duplicate analyses as follows:-

$$\text{estimate of S.D. ("s")} = \sqrt{\frac{\Sigma d^2}{2N}}$$

where d is the difference between the two results in a duplicate determination and N is the number of duplicate determinations performed. Table 15 summarises the values of "s" calculated in this way for duplicate analyses on pooled cord blood while carrying out recovery experiments to determine the accuracy of the method, and for the duplicate analyses on blood from pregnant women from the 12th week of pregnancy given in Section III. The results are given for various levels of oestrogen concentration and are expressed as microgrammes per 100 ml. whole blood.

TABLE 15

Precision expressed as estimates of S.D. ("s") of results from their means (from duplicate determinations).

6

	source of blood	No. of dupl. determinations	oestrogen conc. µg/100 ml blood	"s" µg/100 ml blood.
oestriol	pregnant women	37	0 - 1.0	0.13
	" "	91	1.1 - 10.0	0.22
	umbilical cord	16	50 - 90	5.8
oestrone	pregnant women	71	0 - 1.0	0.08
	" "	66	1.1 - 7.0	0.19
	umbilical cord	21	0 - 1.5	0.21
oestradiol	pregnant women	92	0 - 0.5	0.06
	" "	44	0.6 - 1.3	0.08
	umbilical cord	21	0 - 1.6	0.17

The "s" values for oestriol and oestrone increased with increasing oestrogen concentration but there was only a very slight increase in the values for oestradiol. The "s" values for all three oestrogens were higher for cord blood extracts than for extracts of blood from pregnant women.

A number of useful factors can be calculated from this estimate of the precision of the method. For example, the smallest amount of oestrogen which is distinguishable from zero can be calculated as $\frac{ts}{\sqrt{N}}$; the least amount of oestrogen which is determined with an error of say ± 25% is given by the formula $\frac{100ts}{25\sqrt{N}}$; and the fiducial range of a results can be calculated as $M \pm \frac{ts}{\sqrt{N}}$, where M is the mean of N determinations and t is Student's "t" ("Student", 1908). When analyses are performed in duplicate and the probability, P, = 0.05, then t is 1.96, and these calculations give the values shown in Table 16.

TABLE 16.

Sensitivity and fiducial range calculated for a duplicate from "s" (P = 0.05). (values in μg/100 ml. blood)

Pregnancy blood	Oestriol	Oestrone	Oestradiol
Smallest amount distinguishable from zero.	0.18	0.11	0.08
Smallest amount measured with error of less than ± 25%	0.72	0.45	0.34
Fiducial Range, conc. 1.0 μg/100 ml. or less	M±0.18	M±0.11	M±0.08
" greater than 1.0 μg/100 ml.	M±0.31	M±0.27	M±0.11
Umbilical cord blood			
Smallest amount distinguishable from zero	8.0	0.3	0.2
Smallest amount measured with error of less than ± 25%	32	1.2	0.8
Fiducial range	M±8.0	M±0.3	M±0.2

These values are of great importance when assessing the results of the fractionation of blood oestrogens and the results of the clinical applications of the method.

However it should be remembered that, while these calculations give an indication of the basic handling errors of the method, they do not take into account the errors due to incomplete extraction or any lack of specificity of the method.

3. Specificity.

(a) The Specificity of the Kober reaction.

The pink colour with an absorption maximum at approximately

515 mμ formed in the Kober reaction is a highly specific property of the natural oestrogens. The production of this colour by phenolic fractions which have been further purified by methylation and separated by chromatography into oestriol, oestrone and oestradiol fractions, is good evidence that it is produced by these oestrogens. When applied to urine extracts, however, the pink Kober colour is often masked by yellow-brown colours produced in the Kober reaction by impurities which may be present in relatively large amounts. Correction is made for these interfering colours by the spectrophotometric method of Allen. The validity of this correction depends on the assumption that the wavelength absorption curves of the impurities are linear over the range 480-556 mμ, a condition which is difficult to prove for all extracts. As was stated earlier in Section II,A,2, when describing the 'micro'-Kober reaction, the ratio observed D_{max}/ corrected reading is approximately 0.75 for pure oestrogen methyl ethers when the Kober reaction is carried out as described by Brown (1955), and the optical densities at 516 and 518 mu. of the impurities can be calculated from the formula:

D impurities = observed D_{max} - corrected reading x 0.75

assuming that the wavelength absorption curves are linear between 480 and 560 mμ.

The optical densities of the impurities present were calculated in this way for each of the standard readings given in Table 5 and for a considerable number of the blood extracts derived in Section III. The results are given in Table 17.

There were considerable variations in the amounts of impurities

present, even for replicate determinations on the same sample. However, the mean values for each group in Table 17 show that, on the average, there were practically no impurities present when the pure standards were evaporated from 0.4 ml. of ethanol, but when the standards were evaporated with the solvents used for chromatography, the amounts of impurities present were the same as those found in blood extracts. Apparently the Kober chromogenic impurities in the blood extracts are derived from the solvents used in the method and not from the blood itself. When the standards were added to the solvents, the amounts of impurities tended to increase with increasing oestrogen concentration; however only in the case of oestriol was this increase significant and this could have been due to a small error in the factor, 0.75, used in the calculations. Since the impurities arise from the solvents used and not from the blood itself, the spectral characteristics can be more readily defined. As mentioned earlier, the addition of solvents to the blank tubes is not a satisfactory method of eliminating the colours produced by the impurities, owing to the variability in the amount of impurity present which frequently causes negative optical density readings.

Within the limits of the precision of the method the impurities have linear absorption spectra between 480 and 560 mμ (see Figures 9, 10 and 11). These will be discussed later. Allen's spectrophotometric correction is therefore a valid procedure for eliminating their effect and under these conditions the Kober colour reaction confers a high degree of specificity on the results obtained.

TABLE 17

KOBER-CHROMOGENIC IMPURITIES IN OESTROGEN FRACTIONS

	Calculated optical densities of impurities at 516-518 mμ. (Mean ± standard deviation)		
A. "Pure" Solutions	oestriol	oestrone	oestradiol
Standards without solvents (0.4 μg in 0.4 ml. ethanol)	0.005±0.028 (5)	-0.006±0.030 (8)	0.012±0.022 (16)
Standards with solvents*			
0.1 μg. oestrogen	0.055±0.035 (14)	0.090±0.036 (14)	0.061±0.023 (13)
0.2 μg. oestrogen	0.082±0.035 (14)	0.092±0.034 (14)	0.078±0.034 (13)
0.4 μg. oestrogen	0.075±0.030 (16)	0.101±0.036 (15)	0.081±0.031 (15)
0.8 μg. oestrogen	0.109±0.025 (15)	0.112±0.048 (12)	0.098±0.067 (12)
combined range 0.1-0.8 μg.	0.081±0.036 (59)	0.098±0.038 (55)	0.079±0.042 (53)
B. Extracts from Blood (10 ml.)			
Range of oestrogen content			
0-0.2 μg/10 ml.			0.088±0.037 (154)
0-0.7 μg/10 ml.		0.100±0.039 (147)	
0-1.0 μg/10 ml.	0.070±0.032 (152)		

Number of observations in parenthesis.

* The solvent added to the oestriol tubes was 2.5% ethanol in benzene (7 ml); to the oestrone tubes, 40% benzene in light petroleum (7 ml); to the oestradiol tubes, benzene (5 ml); none was added to the blank tubes.

Several attempts were made to purify the ethanol and benzene. At first the ethanol was purified by refluxing with NaOH and twice distilling. However, there was some evidence that treatment with m-phenylenediamine, as described in the Appendix, decreased the amounts of impurities and this was consequently adopted. Washing the benzene with tap water for 48 hours in a continuous extractor and drying the benzene over $CaCl_2$ before distillation did not decrease the impurities present in the final residue. Another batch of benzene was partially frozen and the liquid portion discarded. After thawing the solid, the procedure was repeated several times until $^1/_3$ - $^1/_2$ of the benzene had been discarded. The remaining benzene was then refluxed with aqueous mercuric acetate for 6 hours to remove thiophene. After refluxing, the benzene was washed thoroughly with tap water and was then fractionally distilled. This procedure was recommended by Ahlers of Unicam, Cambridge as a means of preparing spectrophotometrically pure benzene. However this gave no improvement in the amount of impurity present in the final Kober colours.

The quinol added before evaporation contributes to the impurities. Traces of quinol deposited on the walls of the tube during evaporation and then oxidised are probably a major source of the contaminating chromogens. This is supported by the fact that there are fewer impurities present when the standards are evaporated from 0.4 ml. ethanol. Under these conditions the quinol would not be deposited over such a large area of the tube as when larger volumes of solvents are used and the ethanol would evaporate more rapidly than the larger volumes of benzene so that a smaller surface area of quinol is exposed for a shorter time to oxidation.

During the development of the 'micro'-Kober reaction (see Section II, p. 30), the optimum amount of quinol was found to be 0.5 mg. This concentration gave the most consistent readings with the least amount of impurity. Larger amounts of quinol led to more impurities in the final Kober colour.

(b) Absorption curves of the Kober colours.

Examples of the absorption spectra of pure oestrogen methyl ethers, of "pure" benzene and of extracts from cord blood and peripheral blood from non-pregnant and pregnant subjects are shown in Figures 9, 10 and 11.

From the figures it can be seen that the wavelength absorption curves of extracts of blood from non-pregnant subjects and "pure" benzene are practically linear between 480 and 560 mμ. Since the extracts from non-pregnancy blood should contain no detectable oestrogens these absorption spectra represent the Kober chromogenic impurities present. On this basis, therefore, the Allen spectrophotometric correction is a valid method for eliminating the interference due to the impurities. The absorption spectra of pure oestrogen methyl ethers with added solvent and of blood extracts containing the same amounts of oestrogens are identical, which supports the earlier findings (see Table 13) that the impurities do arise from the solvents and not from the blood. The wavelength absorption curve of the oestriol extract from cord blood was obtained using the 'macro'-Kober reaction and is identical to that of pure oestriol methyl ether also obtained using the 'macro'-Kober reaction, indicating that there are practically no impurities present in the oestriol extracts from cord blood.

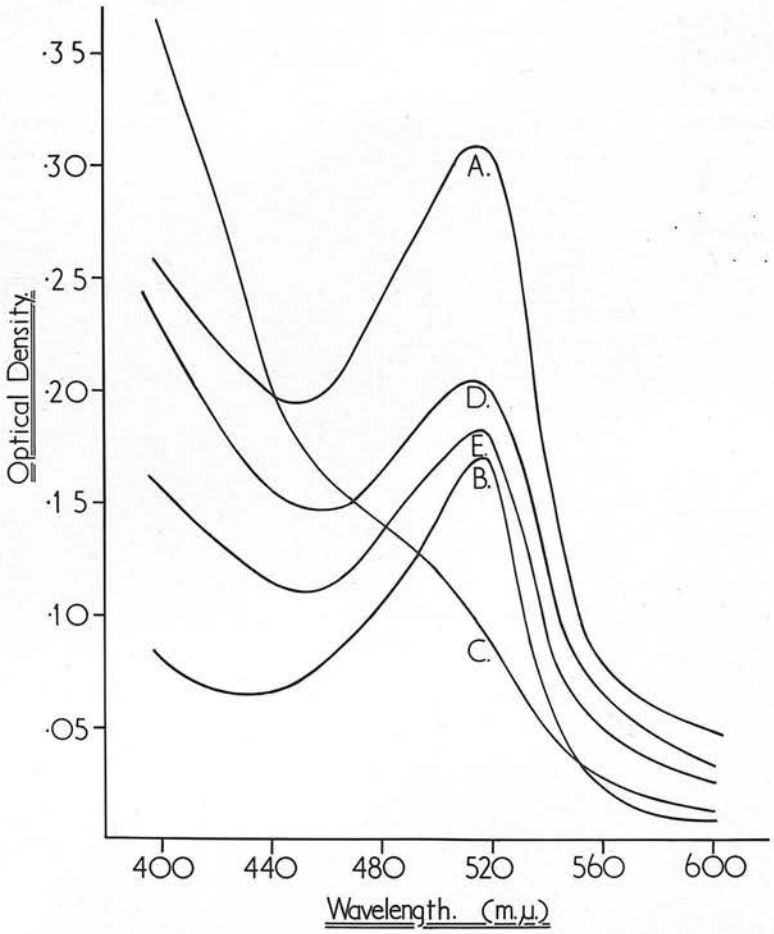


Fig. 9. Absorption spectra of the colours produced in the 'micro'-Kober reaction by A, oestriol fraction from pregnancy blood; C, oestriol fraction from non-pregnancy blood; D, 0·4 μg. oestriol methyl ether in 7 ml. ethanol-benzene; E, 0·4 μg. oestriol methyl ether in 0·4 ml. ethanol; and B, the absorption spectrum of the colour produced in the 'macro'-Kober reaction by the oestriol fraction from cord blood.

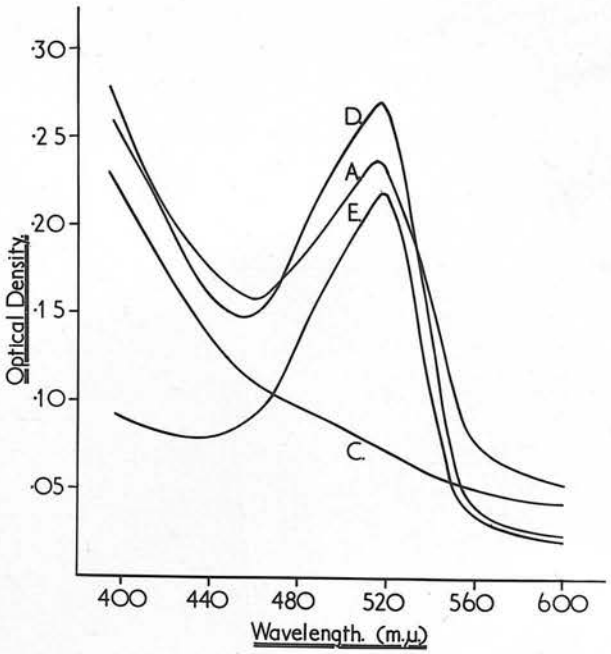


Fig. 10. Absorption spectra of the colours produced in the 'micro'-Kober reaction by A, oestrone fraction from pregnancy blood; C, oestrone fraction from non-pregnancy blood; D, 0·4 μg. oestrogen methyl ether in 7 mls. light-petroleum/benzene; E, 0·4 μg. oestrone methyl ether in 0·4 ml. ethanol.

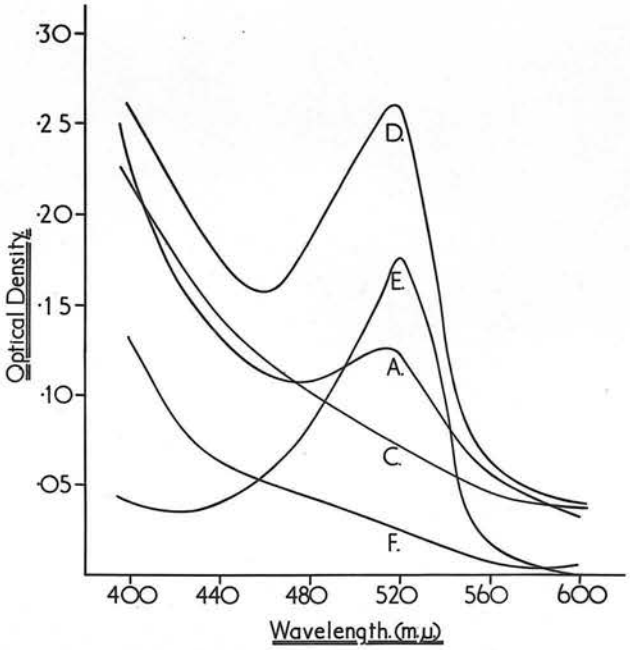


Fig. 11. Absorption spectra of the colours produced in the 'micro'-Kober reaction by A. oestradiol fraction from pregnancy blood; C, oestradiol fraction from non-pregnancy blood; D, 0·4 μg. oestradiol methyl ether in 6 ml. benzene; E, 0·4 μg. oestradiol methyl ether in 0·4 ml. ethanol; F, 6 ml. benzene.

(c) Chromatographic behaviour of blood extracts containing oestrogens.

The elution characteristics of pure oestrogen methyl ethers were given in Section II p.38. The Kober chromogens which are measured as oestriol, oestrone and oestradiol after applying the Allen correction formula have the same absorption and elution characteristics as the corresponding pure oestrogen methyl ethers. Examples are shown in Table 10 in Section II p.38a This is additional evidence that the Kober chromogens which are measured as oestriol, oestrone and oestradiol are in fact these oestrogens and that the method is specific.

(d) Additional purification procedures.

Oestrone and oestradiol fractions from umbilical cord blood were subjected to further purification by the saponification step devised by Bauld (1956) and modified by Brown, Bulbrook and Greenwood (1957a). The oestrone fraction from umbilical cord blood was also separated and purified by formation of the complex with Girard's reagent T, as described by B rown and Blair (1960). The results of the incorporation of these additional purification steps are given in Tables 11 and 12 in Section II, pp.38- 40 .

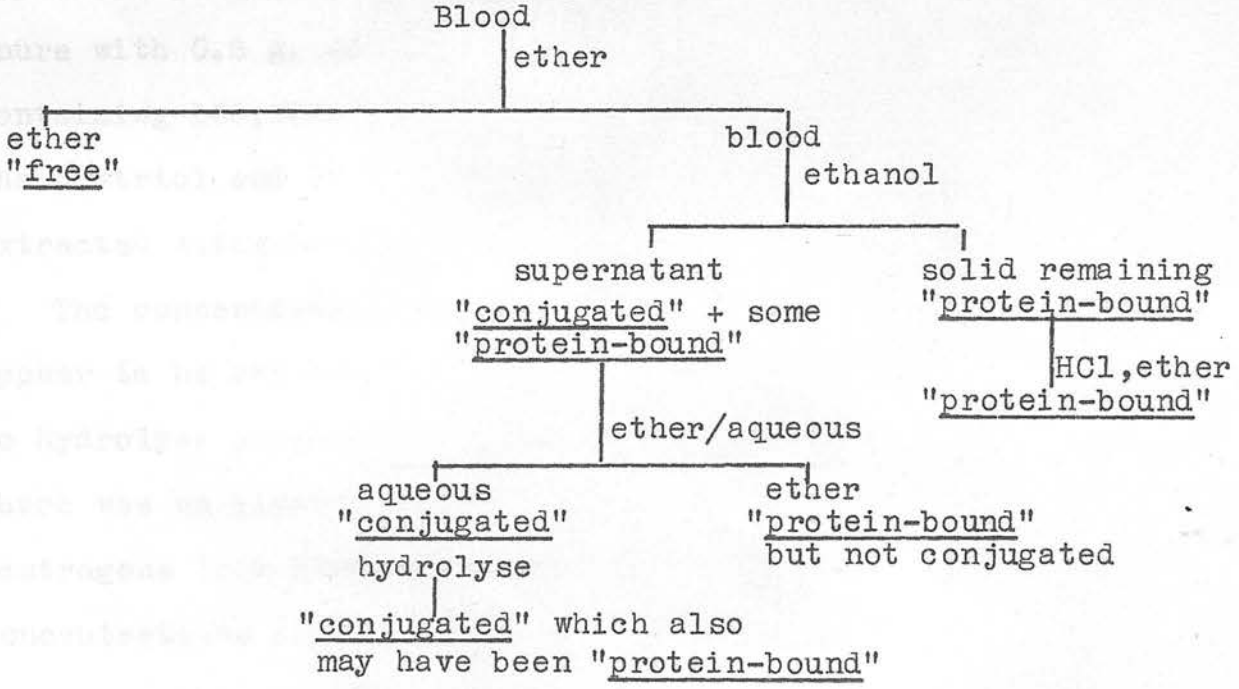
In no instance did the inclusion of either of these purification procedures give purer extracts or results which were significantly different from the results obtained using the unmodified method. Therefore, although saponification and the Girard step remove impurities which interfere in the Kober reaction from urine extracts, they do not remove impurities from blood extracts. If impurities were interfering in the results obtained by the method

after applying the Allen correction, these additional purification steps might have removed them and altered the results. The fact that no change was detected is further evidence that the method is specific for oestriol, oestrone and oestradiol.

E. Extraction of oestrogens from Blood

1. Definition of various forms of circulating oestrogens.

The various forms in which the oestrogens may be present in blood have already been considered briefly. The nature of these forms is defined arbitrarily by the procedure required for their extraction as follows:- 1) free oestrogens can be extracted from aqueous solution with ether and with alcohol; 2) conjugated oestrogens can be extracted from aqueous solution with alcohol, but with ether only after hydrolysis; 3) protein-bound oestrogens can be extracted from aqueous solution partly with alcohol, or with ether after denaturation by boiling with acid. On this basis direct extraction of diluted blood with ether removes the free oestrogens from blood; extraction of blood with alcohol removes the conjugated oestrogens and some of the protein-bound oestrogens; and boiling with acid and extraction with ether removes the remainder of the protein-bound oestrogens. This is summarised in the following scheme:-



2. Methods of Extraction and Results.

a) Direct Extraction with ether

(i) Diluted blood; untreated, boiled with HCl, or hydrolysed with β-glucuronidase.

Several specimens of blood (10 ml.) were diluted to 200 ml. with water and were extracted with ether, either directly or after treatment with hydrochloric acid. The results of these experiments are summarised in Table 18. The yield found after boiling with 30 ml. concentrated HCl (11 N), (i.e. 15 vol. %) for 60 minutes is taken as 100%; if no experiment was performed with 15 vol. % concentrated HCl then the result of boiling with 10 vol.% for 60 minutes was taken as 100%, since little difference was found between the yield obtained by the two concentrations of acid.

Ether extraction of unhydrolysed blood (irrespective of the pH of the blood) removed only some 10-20% of the oestriol and 70-80% of the oestrone and oestradiol. These figures were not appreciably altered by incubating the diluted blood at 37° for 72 hours with 0.2 g. acetone-dried powder from Patella vulgata containing 100,000-140,000 units β-glucuronidase, when only 5% of the oestriol and 80-90% of the oestrone and oestradiol could be extracted with ether.

The concentration of HCl used for acid hydrolysis did not appear to be critical. While 15 vol.% concentrated HCl is necessary to hydrolyse urinary oestrogen conjugates (Brown and Blair, 1958), there was no significant difference in the yields of all three oestrogens from blood when hydrolysis was performed with acid concentrations ranging from 2.5 to 20 vol.% concentrated HCl. This

TABLE 18

Effect of Concentration of Acid on the Amounts of Oestrogens extracted from blood with ether

	Yield (boiling with 15 vol.% taken as 100%)								
CORD BLOOD	Oestriol			Oestrone			Oestradiol		
Treatment									
(a) None	17	(13-19)	(1)	73	(38-107)	(4)	84	(67-100)	(2)
(b) Acidified with HCl (no heating)	11	(8-13)	(2)	47		(1)	103	(50-155)	(2)
(c) Boiled 60 mins with:									
2.5-5 vol% HCl	96	(89-102)	(2)	108	(94-122)	(2)	116	(93-138)	(2)
10 " "	108	(100-127)	(6)	100	(100-103)	(6)	96	(69-106)	(6)
15 " "	100		(2)	100		(2)	100		(2)
20 " "	105	(96-122)	(4)	92	(84-103)	(4)	93	(85-100)	(4)
40 " "	87		(1)	93	(75-100)	(2)	74		(1)
(d) Incubated with β-glucuronidase	5	(2-9)	(3)	83		(2)	90		(2)
MATERNAL BLOOD									
(a) None	10		(11)				114		(1)
(b) Acidified with HCl (no heating)	7	(3-22)	(3)	78	(71-86)	(4)	106	(100-118)	(3)
(c) Boiled 60 mins with:									
2.5-5 vol% HCl	49		(1)	75	(72-77)	(2)	73	(72-73)	(2)
10 " "	87	(72-100)	(6)	99	(92-100)	(5)	85	(63-100)	(4)
15 " "	100		(8)	100		(6)	100		(7)
20 " "	102	(91-118)	(4)	94	(88-100)	(2)	89	(73-100)	(3)

The figures in parentheses give the number of experiments performed with a minimum of 2 determinations for each acid concentration.

was not due to an increased yield being masked by an increased destruction at higher acid concentrations since the recovery of oestrogens added before hydrolysis was unaffected by acid concentration (see Table 19). As stated previously, with 2.5 and 5 vol. % HCl, great difficulty is encountered in achieving complete separation of the aqueous and ether layers. The pigment appears to be evenly distributed throughout the two phases and it is almost impossible to separate any of the aqueous phase prior to centrifugation. Even after centrifuging, the separation is not very satisfactory. However, blood which has been hydrolysed with 15 vol.% concentrated HCl is more easily handled than specimens which have been treated with lower concentrations of HCl and this is the main reason for adopting this acid concentration.

(ii) Recovery of oestrogens added to blood before hydrolysis with different concentrations of HCl.

Several recovery experiments were performed on a sample of pooled cord blood, the oestrogens being added to the blood before boiling with varying acid concentrations. The results are summarised in Table 19.

TABLE 19

Recovery of oestrogens added to cord blood before boiling with varying acid concentrations for 60 minutes.

Concentration of acid used.	No. of experiments.	OESTRIOL µg added per 10 ml.	OESTRIOL % Recovery	OESTRONE µg added per 10ml	OESTRONE % Recovery	OESTRADIOL µg added per 10ml	OESTRADIOL % Recovery
2.5 vol.%	2		56		56		40
5 vol. %	2	16 µg	59	0.4 µg	78	0.2 µg	88
10 vol. %	4		75		60		65
15 vol. %	2		79		54		45
20 vol. %	2		69		64		55

Results are corrected for endogenous blank levels.

While the recoveries are lower when the blood was hydrolysed with 2.5-5 vol. % HCl, the recoveries lie in the range found when hydrolysis is carried out with 15 vol. % HCl (see Table 14 of recoveries). The slightly lower recoveries at the lower acid concentrations may well be due to the increased emulsions formed. Since there does not appear to be increased destruction of added oestrogens with increasing acid concentration, the use of 15 vol. % HCl for routine use is justified.

b) Extraction of blood with ethanol-ether and ethanol.

Ten millilitres of blood were added dropwise to 6 volumes of a mixture of 3 parts ethanol and 1 part ether, and stirred with a mechanical stirrer for 20 minutes. After centrifuging, the ethanol-

ether layer was decanted and evaporated to as small a volume as possible, which was then diluted to 200 ml. with water and boiled with 15 vol.% concentrated HCl for 1 hour. The protein precipitate was also taken up in 200 ml. water and hydrolysed similarly with 15 vol.% concentrated HCl. In other experiments, 10 ml. blood were added dropwise to 100 ml. ethanol and kept at 4°C. overnight. After centrifuging, the precipitate was stirred with a further 30 ml. alcohol and then centrifuged. The combined supernatants were then evaporated almost to dryness. This residue and the protein precipitate were both treated as described for the ethanol-ether treatment. The procedure of Diczfalusy and Magnusson (1958) was followed in a few experiments. Two and a half grammes of $(NH_4)_2SO_4$ were added to 10 ml. blood and the whole was stirred mechanically for 20 minutes with 6 volumes 80% ethanol. The mixture was kept overnight in the deep freeze and then centrifuged.The alcohol layer was decanted off and the precipitate was extracted twice with 10 ml. absolute alcohol. The alcohol supernatants were combined and evaporated just to dryness. This residue and the protein precipitate were both treated as described for the ethanol-ether treatment. The alcohol was not evaporated under an atmosphere of nitrogen as described by Diczfalusy and Magnusson. Compared with acid hydrolysis of whole blood, very low total yields were obtained by this method. However, when oestrogens were added to the protein-ammonium sulphate precipitate before boiling with acid, the recovery was only 24-30% and when the oestrogens were added to these fractions after hydrolysis the recovery was only 43-49%. Therefore the low total yields by the $(NH_4)_2SO_4$- ethanol procedure were probably due to destruction during hydrolysis, and perhaps also at later stages of

of the method. The results are summarised in Table 20.

Eighty to ninety per cent of the oestrone and oestradiol was in the free form; small amounts of oestrone and oestradiol were extracted from the protein-precipitate but these were disregarded since the error in their measurement was large. Sixteen to eighteen per cent of the oestriol was free, which agrees with the results in the preceding section. A further 30-40% of the total oestriol was extracted from the blood by alcohol but was not ether-extractable until after acid hydrolysis or hydrolysis with -glucuronidase. The protein precipitate yielded a further 30-40% on acid hydrolysis. Only 3% of the oestriol was present in the protein fraction when the proteins were precipitated with $(NH_4)_2SO_4$ and ethanol, but in that case the total oestriol extracted was only 43% of that extracted after direct acid hydrolysis, the remainder probably being destroyed during the procedure.

3. Distribution of Oestrogens between Plasma and Red Cells.

Sandberg and Slaunwhite (1957) and Wall and Migeon (1959) showed that up to 20% of oestrone-16^{14} added in vitro to blood was absorbed onto the red cells and that this could be removed by successive washings with plasma or saline. The percentage absorbed was not altered by temperature (0°,22° or 37°) or time, the process being instantaneous. Because the process is instantaneous, it is not possible to determine the percentage attached to the red cells in vivo, since it would occur immediately the blood was removed from the body.

A few experiments were carried out to determine whether these findings could be confirmed by the present method. 40 ml. blood

TABLE 20

Fractionation of Blood with Alcohol or Alcohol-Ether

	Yield (Hydrolysis of whole blood = 100%)		
CORD BLOOD	Oestriol	Oestrone	Oestradiol
Alcohol-ether			
extractable with alcohol and free	18% (15-24) (4)	97% (93-100) (2)	82% (88-95) (2)
extractable with alcohol and conjugated	31% (15-40) (4)	23% (1)	34% (31-38) (2)
bound to protein	36% (30-44) (5)	- *	- *
Total	89% (63-100) (5)	92% (60-100) (4)	113% (84-133) (4)
Alcohol			
extractable with alcohol and free	16% (8-21) (4)	97% (2)	85% (1)
extractable with alcohol and conjugated	35% (21-45) (4)	92% (4)	-
bound to protein precipitate	29% (11-54) (4)	- *	- *
Total	91% (85-100) (4)	92% (60-110) (4)	85% (1)
Alcohol & $(NH_4)_2SO_4$			
extractable with alcohol and free	9% (1)	62% (4)	60% (4)
extractable with alcohol and conjugated	39% (20-59) (4)	65% (1)	78% (1)
bound to protein precipitate	3% (1-4) (2)	-	-
Total	43% (25-61) (2)	67% (47-65) (5)	63% (50-78) (5)
MATERNAL BLOOD			
Alcohol-ether			
extractable with alcohol and free	20% (15-27) (3)	109% (66-134) (3)	95% (90-100) (2)
extractable with alcohol and conjugated	25% (15-38) (3)		
bound to protein precipitate	41% (16-65) (3)	-	-
Total	81% (67-100) (3)	116% (3)	95 (90-100) (2)

* Small positive readings equivalent to less than 0.3 µg. oestrogen per 100 ml. were sometimes obtained, but these were about the limits of sensitivity of the method.

Figures in parenthesis refer to the number of experiments. In the case of extraction with alcohol, the oestrone and oestradiol concentrations were not determined since it appears that they are readily extractable from blood with organic solvents.

were obtained from each of 3 women in the last trimester of pregnancy. In addition five pools of blood from patients in the last trimester of pregnancy were collected, making a total of 8 comparisons.

Half of each of the eight blood samples were centrifuged and the plasma and red cells were separated. In three instances, after separating the plasma and red cells, the red cells were suspended in 0.9% NaCl (volume equivalent to the plasma volume) and centrifuged, and the saline washes were added to the plasma. The plasma, or combined plasma and saline washes of the red cells, and the whole blood were then each diluted to 200 ml. with water and hydrolysed by boiling with 15 vol.% concentrated HCl for 1 hour, and their oestriol, oestrone and oestradiol content measured. The results are summarised in Table 21.

TABLE 21.

Concentration of Oestrogens in Plasma and Whole Blood.

Oestriol		Oestrone		Oestradiol	
Blood	Plasma	Blood	Plasma	Blood	Plasma
4.3	6.1	3.0	2.7	0.6	0.7
3.6	3.4	1.4	1.2	-	-
3.9	3.5	1.9	1.5	0.7	0.5
1.0	1.0	0.5	0.5	0.6	0.5
5.8	5.8	4.3	4.0	1.0	0.9
14.6	*15.7	3.9	*4.0	1.4	* 1.8
1.1	* 1.3	0.6	*0.6	0.2	* 0.2
4.2	* 4.3	0.7	*0.6	0.7	* 0.8

All concentrations in µg/100 ml. whole blood.
Only single determinations carried out
* Red cells washed with NaCl and washings added to plasma.

In 6 instances the red cells were diluted to 200 ml. water and

hydrolysed by boiling with 15 vol.% concentrated HCl for 60 minutes and their oestriol, oestrone and oestradiol content measured (see Table 22). Traces of oestriol were found in the red cell fraction.

TABLE 22.

Concentration of Oestrogens in Red Cells.

Oestriol µg/100 ml.	Oestrone µg/100 ml.	Oestradiol µg/100 ml.
0.3	0.2	zero
0.2	0.1	0.1
0.3	0.2	0.2
0.4	0.2	zero
0.9	zero	zero
0.6	0.2	zero

Red cells washed with 0.9% NaCl.

especially if the red cells had not been washed with saline. No significant amounts of oestrone or oestradiol could be detected in the red cell fraction.

According to the criteria listed in Table 16, no significant difference was found between the oestrogen concentrations in plasma and whole blood. Wall and Migeon (1959) showed that successive saline washes removed all the oestrone from the red cell fraction and that while the first was the most effective, several washes were needed to effect complete removal. On this basis one would expect slightly lower figures for plasma, but in fact the reverse was sometimes observed, although the differences were not significant. It has already been shown in Section II, p. 42, that no changes in oestrogen concentration occur when

blood is stored at 4°C. for up to 5 days before treatment. There is therefore no justification for the extra labour involved in separating off plasma and red cells. Furthermore, blood from the umbilical cord is frequently badly haemolysed and separation of plasma from such speciments is extremely difficult. In addition, the emulsions formed during ether extraction of hydrolysed plasma are often more difficult to handle than those from hydrolysed blood. On this basis whole blood is preferable to plasma for the determination of blood oestrogens.

4. Discussion of Results.

Within the limits of the precision of the method, and taking into account the small number of experiments performed, it seems that most, if not all, the oestrone and oestradiol is present in the free form. A small increase in oestrone and oestradiol concentrations was observed when the diluted blood was boiled with acid before it was extracted with ether, and small amounts of these oestrogens (less than 0.3 µg.per 100 ml. blood) were frequently extracted from the protein fraction after precipitation with ethanol-ether or ethanol. Although these amounts are distinguishable from zero (see Table 16) they were disregarded since they represented such a very small percentage of the total oestrogen present, and the error of measurement as calculated from the precision data was large. However since conjugation probably takes place in the liver, it is highly probable that there will be at least small amounts of conjugated oestrone and oestradiol in blood. Elucidation of this problem awaits the development of more sensitive methods for the detection of these oestrogens.

The situation with oestriol is somewhat different. In this case only 10-20% of the oestriol is in the free form, while acid hydrolysis is necessary before the remainder is rendered ether-extractable. Even β-glucuronidase does not appear to hydrolyse the "oestriol complex" in blood, unless the blood has first been treated with ethanol. The acid concentration used for the hydrolysis of oestriol in blood is not critical as it is for the hydrolysis of urinary oestriol. This suggests either that oestriol is present in a conjugated form which is different from that found in urine (which is unlikely) or that most of the oestriol is protein-bound and, like the oestrone and oestradiol, very little is conjugated, the oestriol-protein complex being effectively cleaved by boiling with relatively low acid concentrations.

Fractionation with ethanol-ether or ethanol again indicates that only about 20% of the oestriol is free. A further 30-40% of the oestriol was alcohol soluble, but was apparently conjugated since it was ether-soluble only after acid or enzymic hydrolysis. The "residual protein-bound" oestriol is present in appreciable amounts, i.e. about 30%. In a few experiments β-glucuronidase was as effective as acid in hydrolysing the "oestriol-complex" present in the ethanol extract, indicating that the protein-bound oestriol which had been extracted with ethanol was conjugated with glucuronic acid. This conjugate was not rendered ether-extractable by β-glucuronidase in diluted whole blood. Either the conjugated oestriol must be removed from the protein before the enzyme can hydrolyse the conjugate or the conjugated oestriol is hydrolysed by the β-glucuronidase but is still bound to the protein. From

these results and the results of hydrolysis with different acid concentrations, it appears that the oestriol conjugates in blood are more readily hydrolysed than the urinary oestriol conjugates. Less oestriol was extracted from the protein precipitate when ethanol was used as the precipitating agent than when ethanol-ether was used. Very low total yields were obtained using $(NH_4)_2SO_4$-ethanol as precipitating agent, probably because of destruction during hydrolysis and evaporation of the alcohol and of the ether.

Szego and Roberts (1946) stated that acid hydrolysis was unnecessary for conversion of blood oestrogens into the free state and that alkaline denaturation was sufficient, but this would not hydrolyse any glucuronide present. This has not been confirmed by the present work, which shows that acid hydrolysis is necessary and that oestriol glucuronide is probably present in blood. Szego and Roberts also showed that all the oestrogens in blood could dialyse through a collodion membrane. On the other hand, Slaunwhite and Sandberg (1959) showed that only 77% of oestriol-$16C^{14}$ added to blood dialysed through against saline, while they and Daughaday (1958) showed that oestrogens did not dialyse through Visking membrane from plasma to albumin solutions indicating that the oestrogens are "bound" to the plasma albumin fraction. The 23% of the oestriol which is non-dialysable is of the same order of magnitude as that which is not extracted with ethanol. Presumably this indicates the presence of protein-oestrogen complex in which the association is much stronger and less easily disrupted.

The results of Diczfalusy and Magnusson (1958) do not agree with the above results. It is possible that the addition of ammonium sulphate does aid in disrupting the protein-oestrogen link; on the

other hand from the recovery experiments quoted here it is possible that the protein-bound oestrogen was destroyed during hydrolysis. There is still much to be explained concerning the form in which oestrogens are present in peripheral blood and more sensitive methods of assay will be invaluable in elucidating this problem.

III. CLINICAL APPLICATIONS OF THE METHOD.

III. CLINICAL APPLICATIONS OF THE METHOD.

GENERAL CONSIDERATIONS.

During pregnancy, the placenta is the main site of production of oestrogens and progesterone. While earlier workers (e.g. Brown, 1957) considered that the placenta produces oestrone, oestradiol and oestriol, current theories suggest that oestrone and oestradiol are produced by the placenta and that portion entering the foetal circulation is metabolized by the foetus to oestriol (Diczfalusy and Magnusson, 1958). The oestriol then passes back to the placenta, and together with some of the placental oestrone and oestradiol, reaches the maternal circulation. The three oestrogens are then subjected to the maternal metabolic processes and are finally excreted mainly in the urine, and a small percentage in the faeces. Gallagher and Brown (unpublished data) using C^{14} labelled oestradiol have confirmed the earlier findings of Pearlman, Pearlman and Rakoff (1954), using deuterium labelled oestrone, that the pregnant woman metabolizes oestrogens in exactly the same manner as the non-pregnant subject. The mother therefore metabolizes the oestrone and oestradiol which she receives from the placenta, and excretes in the urine approximately 20% of the amount reaching the maternal circulation as conjugated oestriol, oestrone and oestradiol; about 80% of the oestriol which she receives is excreted in the urine as conjugated oestriol. The oestrogens circulating in the blood are therefore an equilibrium mixture of the oestrogens entering the maternal circulation from the placenta and foetus and their metabolites before they are eliminated in the

urine. Since metabolism appears to be a constant factor, the 24-hour urinary output is a reflection of the amount of oestrogen produced during a 24-hour period by the placenta. The blood levels depend on four factors; the rate at which the oestrogens enter the maternal circulation, the apparent distribution volume, the rate at which they are metabolized and the rate at which they are excreted. The blood levels are therefore a measure of three processes, and also provide an indication of the amounts of hormones reaching the maternal target organs.

Oestriol is the major urinary oestrogen during pregnancy, and its output has been shown to correlate closely with placental function, being low where placental function is impaired. Consequently, urinary oestriol analyses are useful for assessing placental function in toxaemia, the presence of intra-uterine death, and the duration of pregnancy (Lentners, 1958; Kellar et al 1959; ten Berge, 1959; Frandsen, 1960, a. and b; Finkelstein et al 1960). However, the urinary oestriol output fluctuates considerably from day to day, and all workers have stressed the uselessness of single observations. Accurate 24-hour collections over a period of days were necessary to obtain a reliable estimate of the state of the placenta and foetus. On the other hand, the collection of a sample of blood by venipuncture is a relatively simple procedure, and the main purpose of the present work was to determine the significance of oestriol, oestrone and oestradiol measurements made on such a specimen of blood collected at a moment in time.

The following studies were undertaken.

A. Values during pregnancy in normal healthy subjects.

Series A.

The blood oestrogen levels were measured at four-weekly intervals in a series of normal,healthy, primigravid subjects from the twelfth week of pregnancy to term. The blood was taken between the hours of 2 and 4 p.m. while the patients were attending the ante-natal clinic of the Simpson Memorial Maternity Pavilion. This initial study was restricted to a homogeneous group of primigravid women to rule out any possible difference which might occur between first and subsequent pregnancies.

Series B.

A second series of primigravid and parous subjects were studied during the last month of pregnancy in order to determine whether there was a difference in the levels in primigravid and parous subjects. These patients were also out-patients attending ante-natal clinic.

C. Investigation of variations in blood oestrogen levels throughout the day and from day to day.

A study was made on four subjects to determine whether blood oestrogen levels varied throughout the day and from day to day. Blood samples were taken at 8 a.m., 3 p.m. and 11 p.m. on three consecutive days from four patients.

D. The relationship between blood and urinary oestrogen concentrations.

The relationship between the oestrogen levels in blood and urine was studied by comparing blood and urine concentrations in a series of individuals.

E. The relationship between maternal and foetal levels at birth by Caesarean section.

In the course of the development of the method it was observed

that blood from the umbilical cord (i.e. foetal blood) contained very large amounts of oestriol and very little oestrone and oestradiol, while maternal blood contained much less oestriol and more oestrone and oestradiol. The relationship between blood oestrogen levels in the mother and foetus was therefore investigated.

Series 1.

Samples of blood were taken from the umbilical vein, umbilical arteries and the maternal uterine and arm vein at thirteen Caesarean sections, the peripheral venous blood being removed before administration of the anaesthetic. The oestrone values found in this series were markedly lower than those of the normal Series A. and B, suggesting that the patients delivered by Caesarean section might not be normal from the endocrine point of view. Because of this difference, a second series of subjects was studied to determine whether this difference was due to the effects of drugs given before operation.

Series 2.

Blood samples were taken from ten patients scheduled to have elective Caesarean sections on the day before operation and again immediately prior to administration of the anaesthetic.

F. Blood oestrogen levels in pre-eclamptic subjects.

Blood samples were obtained from some twenty patients with moderate to severe pre-eclampsia.

G. Blood oestrogen levels in non-pregnant subjects.

The oestrogen concentration in blood from non-pregnant subjects was determined in six cases.

H. Comparison with results of other workers.

The results obtained in the original normal series are compared with results published by several other workers for blood oestrogen concentrations at term, and the levels in foetal blood.

Method.

Twenty millilitre blood samples were taken from the arm vein, and duplicate analyses performed on the samples. The amounts of blood obtained from the umbilical vein and arteries were usually less than 10 ml. so that these determinations were done singly. In several instances the volume of umbilical arterial blood was insufficient for measuring the small amounts of oestrone and oestradiol, but was sufficient for measuring the relatively large amounts of oestriol present.

A1) Serial Determinations in Normal Pregnancy.

Venous blood samples (20 ml.) were taken from 12 healthy women at four-weekly intervals from the 12th week of pregnancy to term. If the patient was delivered before the 40th week, then a sample was taken when she was admitted in labour. Two subjects developed mild pre-eclampsia and the results on these (R.F. and M.R.) were excluded when calculating the means and standard deviations for the normal group. Brief clinical particulars are given in Table 23 in Appendix II. The ten normal pregnancies were uneventful except for a slight swelling of hands and ankles which occurred in a few patients.

Results.

The individual results and the means and standard deviations for each week are given in Table 24 in Appendix II. The mean, maximum and minimum levels are shown graphically in Figures 12, 13, 14 and 15 in Appendix II.

There was a considerable variation between individuals; for example, at term the oestriol levels ranged from 3.8 to 19.6 µg/100 ml., the oestrone levels ranged from 1.1 to 8.4 µg and the oestradiol levels ranged from 0.7 to 1.7 µg per 100 ml.

According to the sensitivity data given in Table 16, oestriol and oestrone could be detected from the 12th week of pregnancy and could be measured with an error of less than ± 25% from the 16th week. In all cases positive values for oestrone were obtained at 12 weeks, while in only one case was there no oestriol detectable at this time. Oestradiol could usually be detected from the 16th week and measured with an error of less than ± 25% from the 24th

week. From the 20th week to term all the oestriol and oestrone values could be measured with an error of less than ± 25%, while many of the lower oestradiol values remained below these limits throughout pregnancy.

From the 12th week to term each individual generally showed a persistent rise in the blood levels of all three oestrogens. In the charts the rise is more marked in the mean and maximum levels than in the minimum levels. The oestriol concentration increased at a steady rate which paralleled that of the oestrone and oestradiol until the 32nd week and then rose more rapidly than the oestrone and oestradiol to term, when a mean value of approximately 10 µg. was reached. This change in the blood concentrations of oestriol relative to oestrone and oestradiol is reflected in the changes in the output found in the urine. For example, the increase in the mean blood oestriol concentration from the 16th week to term was fifteen-fold, whereas there was only a ten-fold increase in the mean oestrone and oestradiol levels during this time. In the case of urinary oestrogens, there is a ten-fold increase in the oestradiol output, a ten to twenty-fold increase in the oestrone output and a fifteen to twenty-fold increase in the oestriol output between the 12th week and term (Brown, 1956). These increases in blood oestrogen concentration and in the urinary output of oestrogens therefore parallel one another closely. While the mean group levels of all three oestrogens show a persistent rise throughout pregnancy, the levels of any one or all three oestrogens found in any one individual can fall, and rise again.

B. Measurements in Parous women.

Samples of blood were taken from ten, healthy primigravid women, 37-41 weeks pregnant and at the same time from ten parous women at the same stage in pregnancy.

Results.

The mean results ± standard deviations are given in Table 25. There were no significant differences between the means of this series of primigravid series and Series A (the second series were collected 10-12 months after the first series); the probability, P, of the differences being due to chance being 0.7, 0.3, 0.5 for oestriol, oestrone and oestradiol respectively. Similarly the differences between Series B. and the parous subjects were not significant, P being 0.5, 0.9, 0.3 for oestriol, oestrone and oestradiol respectively. Direct comparisons can therefore be made between both parous and primigravid subjects with obstetrical disorders and the original series, when studying the levels in such patients, since parity does not itself affect the oestrogen levels.

TABLE 25.

Blood Oestrogen Levels in Primigravid and Parous Women (37-42 weeks pregnant).

Mean ± standard deviation in µg/100 ml.

Series.	No. of Subjects.	Oestriol	Oestrone	Oestradiol
Primigravid, Series A.	9	9.71 ±5.00.	5.44 ±2.36.	1.19 ±0.41.
Primigravid, Series B.	10	8.96 ±3.49.	4.36 ±1.00.	1.34 ±0.44.
Parous subjects B.	10.	8.82 ±3.13.	4.28 ±2.03.	1.16 ±0.24

	Oestriol		Oestrone		Oestradiol.	
	Student's "t"	Probability	Student's "t"	Probability	Student's "t"	Probability.
Series A/B	0.383	0.7,N.S.	1.208	0.3,N.S.	0.746	0.5, N.S.
Series B/parous.	0.944	0.5,N.S.	0.107	0.9,N.S.	1.129	0.3, N.S.

N.S. - Not significant.

C. Variations in Blood Oestrogen Levels throughout the day and from day to day.

Twenty millilitre specimens of blood were taken from four patients at 8 a.m., 3 p.m. and 11 p.m. on three consecutive days.

Results

The results are summarised in Table 26. It is apparent that the differences in the blood oestrogen levels at these different times throughout three consecutive days were small and the levels did not vary in the same manner in each patient or on each day. Analysis of Variance was carried out on these figures, and revealed that the differences were not significant. The results of the Analysis of Variance are summarised in Table 27.

A wide scatter of results was found in normal, pregnant subjects (see Table 24, and Figures 13, 14 and 15, Appendix II). Since, in general, there is no significant diurnal or daily variation, this range of values must be due to individual differences in the amount of oestrogen produced in the body and perhaps also to individual variations in the rates of metabolism and excretion of the oestrogens, and not to day to day variations within each patient.

TABLE 26.

Daily and diurnal Variations in the Concentrations of Blood Oestrogens

All values given in μg/100 ml. blood.

Patient	Oestriol				Oestrone				Oestradiol			
	Time	Day I	II	III	Day I	II	III		Day I	II	III	
A.P. 37/52 cervical suture	8 am	6.50	6.00	4.95	2.85	3.60	3.25		1.00	1.00	0.95	
	3 pm	5.20	5.45	3.70	3.15	2.90	2.75		0.85	0.85	1.00	
	11 pm	5.20	6.05	5.20	3.00	2.25	2.15		1.00	0.95	0.90	
B.W.39/52 ante-partum haemorrhage	8 am	4.50	5.05	4.85	1.45	1.75	1.10		0.70	0.75	0.75	
	3 pm	5.15	3.70	4.90	1.80	1.40	1.40		0.65	0.80	0.65	
	10 pm	4.75	4.30	4.25	1.70	1.40	1.15		0.55	0.65	0.60	
M.R. 32/52 thrombo-phlebitis	8 am	5.65	6.95	8.30	1.60	2.20	1.01		0.85	0.85	0.90	
	3 pm	5.30	8.20	6.30	1.80	2.55	1.40		0.85	1.00	0.95	
	11 pm	4.65	7.15	6.05	2.25	2.35	1.30		0.85	0.80	0.90	
J.H.31/52 ante-partum haemorrhage.	8 am	4.00	4.70	4.80	0.95	1.45	1.30		0.60	1.00	0.75	
	3 pm	2.60	3.30	3.00	1.05	1.35	0.95		1.10	0.90	0.70	
	11 pm	4.35	3.80	3.50	1.45	1.30	0.85		0.90	0.80	0.75	

TABLE 27.

Daily and Diurnal Variations - Analysis of Variance

Values for P. the probability.

Patient.	Oestriol		Oestrone		Oestradiol	
	Days	Times	Days	Times	Days	Times
A.P.	< 0.2	< 0.2	*	< 0.2	*	*
B.W.	< 0.2	< 0.2	< 0.2	*	< 0.2	< 0.05
M.R.	< 0.2	> 0.2	> 0.2	*	> 0.2	> 0.2
J.H.	*	< 0.2	> 0.2	*	> 0.2	*

* Variance Ratio less than 1.

D. The Relationship between Oestrogen Concentration of Blood and Urine.

The relationship between the concentration of a compound in blood and in urine is usually expressed in terms of the renal clearance of that compound. The renal clearance is given by the formula:- $C = \frac{UV}{P}$, where C = volume of plasma cleared per minute, U = concentration of the compound in µg/100 ml. urine, V = volume of urine excreted/minute, and P = concentration of the compound in ug/100 ml. plasma. This formula can be applied, however, only when the compound is in the same state in both blood and urine. In the case of the oestrogens being measured here, those excreted in the urine are present as conjugates, while those in blood are present in free, conjugated and "protein-bound" forms. The above formula can therefore be applied only to the conjugates, while the method described here measures total blood oestrogens. As has already been shown (Section II,E), when they are subdivided into their various fractions, the amounts present in each fraction are too small to be measured with any accuracy. Nevertheless as shown in Section II, E, approximately 5% of the oestradiol appears to be conjugated, while 25% of the oestriol is conjugated, a further 40% possibly being protein-bound as well as conjugated. These figures could be used for making approximate calculations of the renal clearance of the conjugated fraction of these two oestrogens. No figures are available for the percentage of oestrone which is conjugated.

Table 28 gives the results of simultaneous oestrogen concentrations in blood and urine and the ratio of total blood

TABLE 28

Relationship between Blood and Urinary Oestrogen Concentrations

Patient	Diagnosis	Urine Vol. ml/hr.	Oestriol			Oestrone			Oestradiol		
			A Blood Concn. µg/ 100ml.	B Urine Concn. µg/hr.	A:B	A Blood concn. µg/ 100ml.	B Urine concn. µg/hr	A:B	A Blood concn. µg/ 100ml.	B Urine concn. µg/hr	A:B
M.B.	bronchitis	203	3.45	908	1:266	0.75	48.3	1:64	0.60	14.3	1:24
L.R.	-	117	4.80	1,058	1:220	0.75	20.3	1:28	0.45	12.5	1:28
J.W.	pneumonia	127	8.30	1,750	1:211	1.10	25.8	1:23	0.81	19.2	1:24
B.W.†	ante-partum haemorrhage	64	3.60	1,224	1:340	1.40	46.4	1:33	0.80	14.4	1:18
		88	5.15	1,280	1:249	1.80	47.1	1:26	0.65	17.6	1:27
		65	4.90	805	1:161	1.40	33.3	1:24	0.65	11.2	1:17
A.S.	ante-partum haemorrhage	275	13.25	3,500	1:264	0.75	25.0	1:33	0.60	18.3	1:31
J.B.	thrombo-phlebitis	115	10.60	1,833	1:173	2.90	45.0	1:16	1.35	19.2	1:14
A.P.	cervical suture	145	5.20	1,125	1:217	3.15	45.0	1:13	0.85	16.7	1:20
J.H.†	ante-partum haemorrhage	58	3.30	711	1:216	1.35	36.6	1:27	1.00	11.6	1:12
		110	2.60	601	1:231	1.05	39.0	1:37	1.10	11.0	1:10
		44	3.00	625	1:208	0.95	30.8	1:32	0.70	9.7	1:14
M.R.†	thrombo-phlebitis	33	6.30	1,013	1:161	1.40	31.4	1:22	0.95	14.8	1:16
		63	5.30	984	1:186	1.80	34.4	1:19	0.85	14.3	1:17
Mean		126	6.53	1,458	1:223	1.51	36.0	1:24	0.82	15.7	1:19

† only first value used when calculating the mean figures.

concentration to urinary concentration in 8 hospitalized patients and one out-patient (L.R.). The ratios remain fairly constant in the same individual and from one individual to another, considering the inaccuracies involved in collecting 2 or 3 hour urine specimens. Since the blood levels remain constant (see Section III C), this indicates that the rate of excretion remains constant. These ratios can be used for speculating on a number of interesting points dealing with the relationship between blood and urinary concentrations. For example, assuming that the renal clearance of oestrogens and the relative proportions of free and conjugated oestrogens are the same in the blood of pregnant and non-pregnant subjects, they can be used to calculate the order of blood oestrogen levels to be expected in the blood of non-pregnant individuals; for example a man with a daily urinary output of 5 µg oestriol, 5 µg oestrone and 2 µg oestradiol would be expected to have blood levels of 9×10^{-4}, 7×10^{-3}, and 4×10^{-3}, µg/100 ml. respectively. These figures are in agreement with the low values found by Svendsen (1960), but are lower than the measurements given by this method, used at the limits of its sensitivity (see Table 33, page 91). Secondly, the values obtained indicate a very high clearance rate for the conjugated oestrogens. If calculations are made using the above figures for the proportion of conjugated oestriol and oestradiol in blood, then the clearance rates would be of the order of 799 and 382 ml/minute for conjugated oestriol and oestradiol respectively. The free oestrogens are relatively small molecules and might be expected to appear also in urine. Their complete absence from urine could be due to protein-binding in

blood, although this does not appear likely since most can be obtained by simple extraction with ether. Another possible explanation is that free oestrogens are present in the glomerular filtrate, but are completely reabsorbed in the tubules, or that they are conjugated in the kidney. This latter explanation is not impossible, for although the liver is considered to be the main site of conjugation, the kidney has been shown to be capable of synthesising glucuronides to a certain extent (Lipschitz and Bueding, 1939).

All these points remain as speculation at present, and further information depends on the development of more sensitive methods for differentiating between the various forms of oestrogen in blood and for measuring their relative concentrations in the blood entering and leaving the kidney.

E. Maternal and Foetal Blood Oestrogen Levels at Birth.

Series 1.

Blood was taken from the umbilical vein and arteries, and from the maternal arm and uterine veins during thirteen Caesarean sections, the maternal sample being withdrawn before administration of the anaesthetic. Specimens of amniotic fluid were also taken at twelve of these operations. One patient was delivered of binovular twins and one of uniovular twins, and in each instance blood was taken from both umbilical cords. All patients were 37-42 weeks pregnant. The clinical particulars of these patients are summarised in Table 29 in Appendix II.

Results on Series 1.

The results of this investigation are summarised in Table 30 in Appendix II. The oestriol concentration was higher in the amniotic fluid than in the umbilical venous or arterial blood in eleven out of twelve cases, while there were no differences in the oestrone and oestradiol concentrations of foetal blood and liquor. Liquor resembled urine in that most of the oestriol was conjugated, only some 4% being free. The concentration of oestriol, oestrone and oestradiol was the same in umbilical venous and arterial blood. The oestriol concentration found in the maternal system (arm vein) was usually considerably lower (5-45%) than that in the foetal system (cord blood), whereas the oestrone and oestradiol concentrations were higher (57-2,000 and 150-1,000% respectively) in the maternal system. In one case, I.D. where the maternal oestriol was 74% of the foetal oestriol level, the patient's membranes had been ruptured 3 days prior to Caesarean section and

the liquor was heavily infected. This might have been the reason for this high figure. In all of the twelve cases from whom a sample of uterine venous blood was taken, the concentration of oestriol was higher in the uterine venous blood than in the peripheral blood, and in 10 and 9 cases respectively, the oestrone and oestradiol concentrations were also higher in the uterine venous blood than in the peripheral venous blood. The mean differences (uterine - arm venous levels) were 1.95 $\pm$ 0.54, 0.53 $\pm$ 0.14 and 0.14 $\pm$ 0.13 for oestriol, oestrone and oestradiol respectively. These differences were highly significant, "t" being 3.59, 3.71 and 3.23 for oestriol, oestrone and oestradiol respectively and P, the probability, being less than 0.01 in each case. This difference can be explained as being due to the loss of oestrogen from the maternal circulation by metabolism and renal excretion, while additional supplies are added via the uterine vein. These differences represent a very high rate of transfer of oestrogens from the placenta to the mother. Taking the uterine blood flow as 600 ml./minute (Browne and Veall, 1953) the mean uterine -arm differences correspond to a transfer of 16,900, 4,500 and 3,600 µg oestriol, oestrone and oestradiol per 24 hours. According to the figures published by Brown (1957) for non-pregnant individuals 56% of the oestriol and 16% of the oestrone plus oestradiol reaching the maternal circulations should appear in the urine; thus 16% oestrone plus oestradiol should be excreted as oestrone, oestradiol and oestriol in the ratio 44: 10: 46, while the oestriol is excreted unchanged. Using these figures and allowing for the different recoveries from blood and urine, these amounts of oestrogens transferred to the maternal circulation

from the foetus should account for the excretion per 24 hours of 15,080; 854 and 195 µg oestriol, oestrone and oestradiol in the urine, which, when all the assumptions and errors are considered, are in satisfactory agreement with the actual urinary excretion of these oestrogens at term.

The mean peripheral levels found in Series 1 were lower than the mean levels found in Series A and B. While the differences in the oestriol and oestradiol values were not significant, the differences between the oestrone values of Series 1 (Caesarean section patients) and Series A were highly significant, "t" being 4.279, P 0.001. Such a difference was unexpected since there were no obvious endocrine abnormalities, the majority of the patients being operated on because they had contracted pelves.

In order to determine whether this discrepancy was due to medication received by these patients prior to Caesarean section, the following experiment was performed.

Series 2.

Twenty millilitre samples of peripheral blood were taken from ten patients on the day before operation and again immediately prior to anaesthetic. These patients all had elective Caesarean sections and had been in hospital for periods of time varying from a few days to two or three weeks.

Results on Series 2.

The results on these patients are summarised in Table 31 in Appendix II. As with Series 1, the differences between the mean oestrone values for Series 2 (Caesarean sections) and Series A were highly significant, "t" being 2.953 and P 0.01. The

differences in oestriol and oestradiol levels in the two series were not significant. The levels found in the two successive days in the patients of Series 2 were not significantly different. The low oestrone values were therefore not due to any pre-treatment such as the administration of atropine a few hours before operation, either directly by decreased production or indirectly by interference in the method of assay.

About this time, it was found that the mean oestrone concentration in a random series of eleven patients who had been admitted to hospital for a variety of ailments such as respiratory infections, thrombophlebitis, and slight ante-partum haemorrhages, was 1.81 µg/100 ml, a figure which is the same as that found in the two series of patients who had Caesarean sections. Since these patients and the patients who had been subjected to Caesarean section were all in bed in hospital, whereas the normal series (A and B) were all ambulant out-patients attending the ante-natal clinic, the possibility arises that the differences in blood oestrone levels are due to the effect of rest in bed. This possibility is now being investigated.

F. Concentration of Blood Oestrogens in Patients with pre-eclamptic Toxaemia.

The concentration of blood oestriol, oestrone and oestradiol was measured in some twenty patients with pre-eclamptic toxaemia. Estimations were performed in duplicate, usually on one sample of blood, but in some cases several samples of blood were collected over a period of days and assayed. The clinical findings and the results in these patients are given in Table 32, and the results are also presented graphically in Figures 13, 14, 15 and 16 in Appendix II. The mean, maximum and minimum levels of oestriol, oestrone, oestradiol and total (i.e. sum of these three) oestrogen levels found in normal subjects are shown with the individual results for the pre-eclamptic subjects superimposed.

Results.

1. The relationship between blood oestrogen levels and the severity of the condition.

The oestradiol concentration in the pre-eclamptic subjects fell within the normal limits except in four patients where the results were lower than normal; in three of these patients foetal death occurred either on the day the blood was withdrawn or the day after, and in the fourth, foetal death occurred some time later. The scatter of results about the normal mean is quite regular, indicating that, in general there was no difference in the oestradiol values found in the normal and pre-eclamptic group.

Except in one patient, all the oestrone concentrations lay below the mean of the normal series, and in eight patients they lay below the normal minimum values. Five of these eight patients were delivered of still-born infants, one was delivered of a child who

lived only a few hours and the eighth was delivered at 37 weeks of a child who weighed only 4 lb.13 oz. but who survived.

The pattern for oestriol and total oestrogens was similar to that for oestrone. Apart from four patients (one of whom had the high oestrone level) all the oestriol concentrations were less than the normal mean values, with the results in six patients lying below the normal minimum values. The other four patients had higher oestriol levels; in one case the oestriol concentration, determined on two separate occasions, was higher than the normal maximum levels found.

The oestriol concentrations in four of the patients delivered of still-born infants were below the normal minimum value. The concentration of total oestrogens usually reflected the oestriol concentration, since oestriol is the oestrogen present normally in the highest concentration in the latter part of pregnancy.

Very low oestrone and oestriol levels appeared to be associated with severe pre-eclampsia of early onset, and with foetal death. These low blood oestrogen levels are probably the result of placental insufficiency giving decreased production of oestrogens. However, in a few cases, the oestriol levels tended to be in the higher range of the normal series. Whether these higher values are due to impaired renal function is still being investigated.

In view of the low oestrone values found in patients about to have Caesarean sections, these results must be viewed with caution until it has been established whether bed-rest has any effect on blood oestrogen levels (see Section III, E).

2. <u>The relationship between blood oestrogen levels and baby weight</u>.

In five instances where the baby weighed less than 5 lb. at

birth, the oestriol values were within the normal range, three being less than the normal mean value, two being just above the normal minimum values; in four women the oestriol concentrations were below the minimum values of the normal series. The oestrone concentrations in all nine cases were below the minimum values for the normal subjects. Of these nine patients, eight were suffering from pre-eclampsia, the ninth was a case of essential hypertension in a subject who had had pre-eclampsia in a previous pregnancy in association with a still-birth. Only two of these nine babies survived, one being that of the patient with essential hypertension. In general, very low oestrogen levels seem to be associated with small babies who have a poor survival rate and with intra-uterine death.

G. Results from non-pregnant subjects.

Six duplicate analyses were performed on blood from non-pregnant subjects. Two of the specimens were obtained from a young woman; the remainder were obtained from single individuals from the Blood Transfusion Unit and were diluted with citrate-dextrose solution, so that 10 ml. blood assayed would correspond to 7.5 ml. donor's blood. The concentrations of oestrogens found in the extracts are summarised in Table 33.

TABLE 33.

Concentration of Oestrogens in Blood from non-pregnant subjects

(all concentrations in µg. oestrogen/100 ml)

	Oestriol	Oestrone	Oestradiol
	0.07	-0.04	-0.03
	0.15	0.03	0.00
	0.11	0.13	-0.05
	-0.30	0.18	-0.18
	0.18	0.02	-0.04
	0.04	0.30	0.05
Mean	0.04	0.12	-0.04
Smallest amount distinguishable from zero.	0.18	0.11	0.08

The mean concentrations of oestriol, oestrone and oestradiol in the six specimens are below the limits of sensitivity of the method. In one instance, the oestriol concentration is just significantly greater than zero ($P = 0.05$), and in three instances, the oestrone values are significant; no oestradiol value is significantly greater than zero; however one oestriol value and

one oestradiol value are more negative than can be explained on the basis of the precision of the method, indicating that impurities giving negative corrected readings are present.

From the urinary output of oestrogens and blood oestrogen concentrations in pregnant women and the urinary oestrogen output in non-pregnant subjects, one would expect very low blood levels of oestrogens outside pregnancy; in fact they should hardly be detectable by the present method. The concentrations found are, on the average, considerably less than those found in the blood of pregnant women after the 12th week of pregnancy and are of the same order as those found in non-pregnant subjects by Preedy and Aitken (1957). The few positive values are just above the limits of sensitivity of the method, where the error of the determination is large. Whether these figures do in fact represent oestrogens has yet to be proved. Preliminary observations in 5 patients using the more sensitive Ittrich fluorescence reaction indicate that these figures are too high, and that the levels in peripheral blood about the time of ovulation are of the order of 0.03 µg. and less of oestriol, oestrone and oestradiol per 100 ml. blood; these are similar to the values calculated from the blood urine ratios and urinary values of Brown (see Section III, D).

The finding of low or zero concentrations of oestrogens in the blood of non-pregnant individuals adds weight to the specificity of the method. Obviously there is no gross contamination of the final fractions with impurities giving positive or negative corrected spectrophotometric readings.

H. Comparisons of blood oestrogen concentrations reported from several laboratories.

Table 34 summarises the blood and plasma oestrogen concentrations in pregnant and non-pregnant subjects reported by several workers. There is good reason to accept the specificity of the methods employed by Preedy and co-workers who identified the oestrogens by fractional gradient elution partition chromatography, and of Oertel et al. who isolated oestriol, oestrone and oestradiol from a pool of blood from women in the last trimester of pregnancy, and identified them by infra-red specroscopy. The recovery rates reported by the various workers were: Preedy and Aitken 80%, Svendsen 65%, Varangot et al. 60-80%, Veldhuis 80-90%. The concentrations found by Varangot et al.(1956) are very much higher than the concentrations reported by other workers. As already stated (Section I), these high results are probably due to the use of a non-specific fluorimetric method of assay with impure blood extracts without any additional check for specificity. The results obtained by the present method are approximately half those obtained by Preedy and co-workers and Oertel et al. for plasma. There is therefore good agreement between the results obtained by the three methods when allowance is made for the fact that the plasma is equivalent to approximately half the blood volume. The oestradiol values quoted by Svendsen (1960) agree with those given by the three methods mentioned above, but his oestrone values are lower and in fact are lower than his oestradiol values in every case. This might be the result of his extracting with chloroform without any previous hydrolysis. The results of Veldhuis (1953), while a little lower, also agree remarkably well when one considers

TABLE 34.

Comparison of Results from different Authors.

	No. of Observations.	Oestrogen Concentration (μg/100 ml. plasma or blood)		
		Oestriol	Oestrone	Oestradiol
Blood from Pregnant Women.				
Varangot et al (1956);blood 8-8½ months	8	24-280 (156)	11-155 (59)	
Veldhuis (1953);plasma, 7-8 months	3	1.0-1.6 (1.4)	2.1-3.3 (2.7)	
Oertel et al(1959);plasma,3rd trimester	1	2.9	5.2	3.3
Preedy & Aitken (1957);plasma 38 weeks -				
Aitken et al. (1958)	11	4.3-19.8 (13.3)	2.7-17.8 (9.0)	0.6-3.7 (2.0)
Roy and Brown (1960);blood 36 weeks -	19	2.3-19.6 (7.8)	0.5-9.0 (5.0)	0.2-1.7 (1.0)
Svendsen (1960);	7	-	0.1-1.0	0.2-3.0
Foetus at birth (Cord Blood)				
Aitken et al (1958); plasma	5	59-229 (129)	1.0-6.1 (3.6)	0.2-0.8 (0.5)
Diczfalusy & Magnusson (1958);blood	9	39-68 (58.3)	zero-2.3(1.3)	0.1-1.3 (0.6)
Roy and Brown (1960); blood	22	30-70 (49)	0.7-2.3 (1.5)	0-1.5 (0.6)
Blood from Non-Pregnant Subjects.				
Varangot et al (1956);blood-women during cycle	39	0-32 (4)	12-190 (73)	
" " blood-men and post menopausal women	38	0	70-100 (110)	
Veldhuis (1953);plasma - pooled	1	0.39	0.31	
Preedy & Aitken (1957);plasma - luteal phase	6	0.15,0.21,0.30	0.06,0.13 0.14,0.22	0.07
" " plasma - men	5	0.15	0.06,0.11	0.07
Roy and Brown (1960);blood - pooled,and during cycle.	6	0.18	0.11,0.13 0.18,0.30	0.08
Svendsen (1960).	5		0.01-0.065	0.025-0.075

the many assumptions underlying the corrections for impurities which Veldhuis used. There is very close agreement between the results for pooled cord blood found by Diczfalusy and Magnusson (1958) and those reported here, while the results of Preedy et al. (1958) are higher, even when allowance is made for plasma volume. Preedy and Aitken found small amounts of oestriol and oestrone in plasma from some non-pregnant subjects. Svendsen found slightly less oestrone and oestradiol. Similar concentrations were found here, but they were so low that the error in their measurement was great and little significance could be attached to them. Whether these figures do, in fact, represent oestriol and oestrone is a matter for further investigation.

IV. SUMMARY AND CONCLUSIONS.

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The fluorescence reaction described by Brown (1952) was reinvestigated for use with 0.05 - 0.1 µg. pure oestrogen and with blood extracts prepared in the same manner as urine extracts as described by Brown (1955). The fluorescence reaction proved satisfactory for the measurement of these small amounts of oestriol, oestrone and oestradiol in pure solution, but it was not specific enough for use with blood extracts prepared by Brown's method. Extracts which should have contained no oestrogens gave as much fluorescent activity as extracts from blood which contained large amounts of oestrogens.

The Kober reaction is a highly specific reaction of the natural oestrogens, but at the time of commencement of this work was normally carried out with several microgrammes of oestrogens in a final volume of reagent of 3-4ml. The 'micro'-Kober reaction was therefore developed so that 0.05 - 1 µg. oestrogen could be measured in a final volume of 0.4 ml., optical density measurements being made in a cuvette with a light-path of 10 mm. The 'micro'-Kober reaction proved to be sufficiently sensitive and specific for use with extracts of blood obtained from the umbilical cord and from the pregnant woman. It was not sensitive enough to measure with any degree of accuracy the amount of oestrogens present in blood from non-pregnant subjects, although on a few occasions small amounts of oestrone were detected in blood from this source. Contamination with dust particles, etc. had to be rigorously excluded from the tubes during the 'micro'-Kober reaction, since this gave yellow colours which interfered in the measurement of the

optical densities. The wavelength absorption curves of the colours developed in the Kober reaction indicated the presence of impurities which were derived from the benzene and benzene-ethanol used for chromatography. The impurities came in part from the quinol and in part from the benzene and the ethanol. Several attempts to purify the benzene were unsuccessful and the smallest amount of quinol necessary to give consistent readings was used. The absorption spectra of extracts of blood from pregnant women were almost identical to those of the corresponding pure oestrogen methyl ethers dissolved in the same volumes of solvents as those used for eluting them from the alumina columns, indicating that the blood extracts contained few impurities other than those derived from the solvents themselves. The absorption spectrum of 'pure' benzene and extracts from non-pregnant subjects were practically linear between 480 and 560 mμ. The use of the Allen correction formula to correct for the optical densities given by the impurities was therefore justifiable. This conclusion was further supported by the following findings. By the use of the Allen correction formula it was possible to calculate the optical densities given by the impurities. This was done for a series of standards (with and without added solvent) and for a series of blood extracts. The amounts of impurities present in the blood extracts were the same as those present in the series of pure oestrogens and solvents.

The method finally adopted was almost identical to that described by Brown (1955) for the measurement of urinary oestrogens. Acid hydrolysis of whole blood proved the most satisfactory means of getting the maximum yields of oestrogens. Diluted, whole blood

was hydrolysed by boiling with 15 vol. % concentrated HCl, the hydrolysed blood was extracted with ether, and the ether extracts were processed exactly as described by Brown, except that chromatography was performed with 0.7 g. alumina and the final measurement was made by the 'micro'-Kober reaction. Troublesome emulsions formed during the initial ether extractions and at partition between benzene/light petroleum and 1.6% NaOH, but these were handled by careful manipulation and centrifugation.

The accuracy of the method was less than that of the urinary method. The recovery of oestrogens added after hydrolysis was 60-70%, and the recovery of oestrogens added before hydrolysis was some 5-20% lower. The sensitivity of the method was such that 0.18 µg. oestriol, 0.11 µg. oestrone and 0.08 µg. oestradiol could be detected in 100 ml. blood. This proved satisfactory for the measurement of oestrogens in blood from women from the 12th week of pregnancy to term.

The problem of extracting oestrogens from blood was investigated. Blood was separated into plasma and red cell fractions, and in some instances the cells were washed with 0.9% saline. The plasma was found to contain practically all the oestrogens present in whole blood although traces of oestriol were sometimes found in the red cell fractions, particularly if the cells were not washed with saline. This is in agreement with the work of Wall and Migeon (1959) who showed that successive saline or plasma washes removed all the oestrogen from the red cell fraction, which they found to contain up to 20% of the total oestrogen content.

Direct ether extraction of whole blood, or extraction with

ethanol-ether or ethanol removed 10-20% of the total oestriol and 80-90% of the oestrone and oestradiol (yield given by boiling with 15 vol. % HCl being taken as 100%). The ethanol or ethanol-ether fraction yielded a further 30-40% of the oestriol on hydrolysis by boiling with HCl or by incubating with β-glucuronidase. The remainder of the oestriol was recovered from the protein precipitate by acid hydrolysis followed by ether extraction. Hydrolysis of the whole blood by boiling with HCl in concentrations varying from 2.5 to 20 vol.% gave the highest yields of oestrogens, the acid concentration not being critical. There was no increase in the amount of oestriol which was extractable with ether after incubation of the blood with β-glucuronidase. These experiments indicate that almost all of the oestrone and oestradiol are in the free form in blood, 10-20% of the oestriol is in the free form, 30-40% is either protein-bound or conjugated or possibly both protein-bound and conjugated, and the remainder is more firmly protein-bound.

Blood oestrogen levels were measured in normal, healthy, pregnant women, patients who were delivered by Caesarean section and patients suffering from pre-eclampsia. There were no differences in the levels in primigravid and parous subjects. There was a very wide scatter of results in the normal patients studied. Since there were no significant diurnal or daily variations in the concentration of blood oestrogens, as judged by the results obtained at various times of the day, and on consecutive days in four patients studied in the last trimester of pregnancy, this scatter was due to real differences in the

individual levels. The concentrations of oestrogens rose from the 12th week of pregnancy to term. This rise was usually continuous, but in some patients the levels sometimes fell temporarily, usually to rise again. The three oestrogens rose at about the same rate until the 32nd week of pregnancy when the oestriol began to increase more rapidly than the oestrone and oestradiol. These rises in blood oestrogen levels closely paralleled the corresponding rises in urinary oestrogen output. Using the mean values in Series A and the figures for urinary output (Brown, 1956) of 5,100; 510 and 140 µg. oestriol, oestrone and oestradiol at 20 weeks, and 33,500; 1,550 and 570 µg. at 40 weeks, the factors for the increases in oestrogen concentrations between the 20th and 40th week were (urine/blood) 6.5/6.0, 3.0/3.3 and 4.1/4.4 for oestriol, oestrone and oestradiol respectively. These calculations showed that the increases in the concentrations of all three oestrogens in urine closely paralleled the increases in blood, even to the extent of the more rapid increase in oestriol concentration after the 32nd week. Although the increases in blood and urine followed one another closely, the ratio of the relative concentrations of oestriol, oestrone and oestradiol were markedly different for blood and urine. Using the same values as in the above calculations, the ratio of oestriol: oestrone: oestradiol were 22:1:0.37 and 1.8:1:0.22 for blood and urine respectively. The difference in ratio of oestriol to oestrone suggested that oestriol is cleared very much more rapidly than oestrone, but, on the other hand it might be the results of the difference in the relative proportions of free, conjugated and "protein-bound" oestriol

and oestrone in blood.

Since the blood levels showed no significant diurnal variation, and the ratio of blood concentration to urinary oestrogen output per hour remained more or less constant, it appears that the rate of excretion of oestrogens must also remain constant. These ratios were used as a basis for speculation on the levels one might expect to find in blood from non-pregnant subjects, and on possible modes of excretion of the oestrogens.

The concentration of oestrogens in amniotic fluid, umbilical venous and arterial blood and uterine venous and arm venous blood of the mother at Caesarean section were measured. Liquor contained very large amounts of conjugated oestriol and only 4% of free oestriol; in other words its oestrogen content resembled that of urine rather than that of blood. There was no significant difference in the concentration of the three oestrogens in umbilical venous and arterial blood. Small differences might have been expected if the foetus were an important site for the metabolism of oestrone and oestradiol (Diczfalusy and Magnusson, 1958). The concentrations in maternal uterine venous blood were significantly higher than those in the maternal peripheral blood, sometimes by as much as 25%. This difference probably represents the gradient caused by entry of oestrogens into the maternal circulation from the placenta and loss through maternal metabolism and renal and faecal excretion of the oestrogens. The fact that a difference is detectable with the high uterine blood flow of approximately 600 ml./minute indicates a considerable production of oestrogens by the placenta. The peripheral oestrone levels were

significantly lower in the women undergoing Caesarean section than in the normal series. This difference was shown not to be due to any premedication administered to the patients, and the possibility that it is due to rest in bed is under investigation.* In support of this possibility it is interesting to note that the blood oestrone levels in a series of patients admitted to hospital with a variety of minor ailments were also significantly lower than those of the normal out-patients and that the urinary output of oestrogens falls overnight in pregnant and non-pregnant subjects (Brown, unpublished observations); similarly Fotherby and Strong (1960) showed that the urinary output of the metabolites of a number of adrenal steroids fell between 9 p.m. and 6.30 a.m.

In the majority of cases of pre-eclampsia, there was a decrease in the concentration of oestriol and oestrone in blood, but little or no change in the oestradiol concentration, except in some cases of intra-uterine death. In all cases of still-birth the oestrone concentration was below the normal minimum and in almost all these cases the oestriol concentration was also below the normal minimum. These low concentrations of blood oestrogens were presumably due to placental dysfunction. In a few cases of severe pre-eclampsia where the symptoms were of short duration and had appeared after the 34th week of pregnancy, the oestriol concentration was above the normal mean value and in one case was higher than the maximum value observed in the normal series,

* Preliminary observations in 5 patients, from whom blood samples were taken on admission to hospital and after 3 days in hospital, show that there is no significant change in the oestriol and oestradiol levels, but that the oestrone levels fall to approximately 50% of their original values after three days in hospital.

although the oestrone values were low. It was suggested that these higher levels might be the result of the renal damage which is known to occur in pre-eclampsia.

The results obtained by using the present method agreed well with those obtained by Preedy and co-workers and Oertel et al, while the oestrone values of Svendsen were lower, but again there was good agreement in the oestradiol values. There was very close agreement between the results for cord blood quoted here and those of Diczfalusy and Magnusson. The specificity of these methods and the fluorimetric methods of Veldhuis and Varangot et al were discussed.

Very small amounts of oestriol and oestrone were found in samples of blood taken from non-pregnant subjects, and the mean values were below the limits of sensitivity of the method. Preliminary observations using the more sensitive fluorimetric method of Ittrich (1958) suggest that the levels are even lower than those indicated by the results of the 'micro'-Kober reaction and are near the limits of sensitivity of the Ittrich reaction.

APPENDIX I.

Reagents and Apparatus

In general, the reagents and apparatus were the same as those described by Brown (1955).

All chemicals were analytical grade. Ether, benzene and light petroleum (b.p. 40-60°) were distilled before use; while the benzene and light petroleum used for chromatography were saturated with water. Ethanol (absolute) was allowed to stand over m-phenylediamine for 1 week, and thrice distilled, the second distillation being carried out through a fractionating column. Ethanol, purified by refluxing with NaOH and twice distilling, was also used for some experiments. The NaOH and $NaHCO_3$ solutions were prepared on a w/v basis. Concentrated carbonate solution (pH 10.5) was prepared by adding 20% NaOH (150 ml.) to 8% $NaHCO_3$ (1 litre). Alumina (Savory and Moore, Ltd., London) was deactivated with 9-10% water to the activity specified by Brown (1955). Sand for protecting the alumina columns during chromatography was cleaned by boiling with 30% HCl, washing thoroughly with tap water, followed by distilled water, and finally with ethanol, and was then dried.

The sulphuric acid reagents used for the fluorimetric reactions were prepared as follows. For the oestriol reagent, 0.2 g. arsenious acid was dissolved in 100 ml. 76% H_2SO_4 (v/v) by warming gently. The oestrone/oestradiol reagent was similarly prepared by dissolving 0.2 g. arsenious acid in 100 ml. 68% H_2SO_4 (v/v).

Kober reagents containing quinol and sulphuric acid were prepared as described by Brown (1955). The oestriol reagent was

prepared by dissolving quinol (2 g.) in 100 ml. 76% H_2SO_4 (v/v) with warming; the oestrone and oestradiol reagents were similarly prepared by dissolving quinol (2 g.) in 100 ml. 66% H_2SO_4 (v/v) and 60% H_2SO_4 (v/v) respectively.

Catechol was purified by vacuum distillation and was stored in a stoppered tube in the deep freeze. The ethanolic solution was made up freshly every fortnight.

Chromatography was performed in 10 mm. sintered glass filter tubes, porosity no. 3, 5 cm. long and sealed on to a reservoir of 25 ml. capacity. The fractions containing the oestrogens were collected in 12 x 1½ cm. tubes fitted with a B14 socket for connection to the evaporator, and narrowed at the other end for a length of 10 mm. to a diameter of 10 mm. The Kober colour reaction was performed in this narrowed section of the tube. The fluorescence reaction was carried out in similar tubes which narrowed to a diameter of 10 mm. for approximately 20 mm. length.

The solvents were removed by evaporation from these tubes as follows. The tubes (four at a time) were connected to a manifold with B14 joints. The manifold was connected to a cylinder of nitrogen and side tubes from the joints led through a second manifold to a water-pump. Solvents were removed under reduced pressure and in an atmosphere of nitrogen (see Section II, 3).

The Kober tubes were cleaned with chromic acid at approximately monthly intervals. They were soaked in chromic acid, were washed thoroughly with tap water, were rinsed with an acid sodium sulphite solution, were washed thoroughly with tap water to remove the sulphite,

and finally rinsed with distilled water.

Optical densities were measured in a Unicam SP600 spectrophotometer using micro-cells with a light-path of 10 mm. and a capacity of 0.4 ml.

The fluorescence intensity was measured in a Farrand fluorometer using Pyrex tubes of 10 mm. diameter and 80 mm. in length. Corning glass filter 5113 was used as primary filter, and Corning glass filter 3384 was used for the secondary filter.

APPENDIX II.

TABLE 23

CLINICAL DETAILS OF PATIENTS IN NORMAL SERIES

Patient	Age	Date of L.M.P.	Date of Delivery	Duration at Delivery	Sex of child	Weight of child lbs.oz.	Placental weight lbs.oz.	
I.B.	31	7.11.58	6. 8.59	39/52	M.	8 2½	1 9	
J.B.	25	19.12.58	1.10.59	41/52	M.	7 13¼	1 7	
S.K.	18	3.11.58	23. 8.59	42/52	M.	6 13½	1 4	Imferon for iron-deficiency anaemia; Folic acid for megaloblastic anaemia of pregnancy
C.M.	35	16.12.58	30. 9.59	41/52	M.	5 9	1 -	
D.M.	33	23.10.58	6. 8.59	41/52	M.	8 8	1 7	
F.R.	25	7.12.58	27. 9.59	42/52	F.	8 -½	1 3	
I.R.	30	28.10.58	23. 7.59	38/52	F.	7 6	1 8	6.6.59-13.6.59 admitted with slight A.P.H. Treated with Imferon for iron deficiency anaemia.
C.T.	25	25.12.58	1.10.59	40/52	F.	7 1	1 9	
G.W.	27	29.11.58	11. 9.59	41/52	M.	7 11	1 9	
M.W.	23	5.11.58	23. 8.59	42/52	F.	6 5¾	1 1	Imferon for iron deficiency anaemia, + Folic acid.
R.F.	22	6. 1.59	16.10.59	40/52	M.	7 3½		Mild pre-eclampsia; Saluric between 32nd and 36th weeks.
M.R.	20	8.12.58	13. 9.59	40/52	F.	6 4		Mild pre-eclampsia; Hydrosaluric 8.7.59 Serpasil 25.7.59; onset about 29th week.

TABLE 24. Serial Results in Normal Pregnancy

Patient	Oestrogen (µg/100 ml)	12	16	20	24	28	32	36	40 weeks
I.B.	Oestriol	0.05	0.35	2.40	3.50	3.65	7.40	12.10	19.60
	Oestrone	0.05	0.50	3.10	3.35	4.35	6.90	4.40	6.20
	Oestradiol	nil	nil	0.25	0.90	0.85	1.10	1.10	1.55
J.B.	Oestriol	0.25	1.40	1.85	2.20	4.20	2.15	6.30	10.70
	Oestrone	0.25	0.35	1.15	2.0	4.20	2.05	4.90	7.0
	Oestradiol	nil	0.10	0.15	0.40	0.50	0.70	0.70	1.50
S.K.	Oestriol	0.05	0.40	1.60	4.40	2.85	1.80	2.30	6.20
	Oestrone	0.10	0.30	0.50	0.70	0.65	2.40	0.50	1.05
	Oestradiol	nil	nil	0.10	0.10	0.10	0.90	0.20	0.65
C.M.	Oestriol	0.15	1.20	1.20	2.00	3.00	2.15	3.45	2.75
	Oestrone	0.15	0.45	0.90	2.00	4.15	2.30	3.10	3.10
	Oestradiol	nil	0.15	-	0.65	1.00	0.90	0.40	1.05
D.M.	Oestriol	0.30	0.60	1.50	2.00	3.30	5.40	10.40	14.20
	Oestrone	0.50	0.75	2.10	3.65	5.15	8.30	6.00	7.90
	Oestradiol	nil	0.20	0.60	0.20	0.20	1.10	1.05	1.60
F.R.	Oestriol	0.45	1.40	2.50	2.70	3.15	5.20	8.95	12.1
	Oestrone	1.25	1.85	3.25	5.50	7.55	6.70	8.95	8.35
	Oestradiol	0.40	0.05	0.55	0.60	1.15	1.15	1.20	1.20
I.R.	Oestriol	0.10	0.25	1.15	2.35	2.20	2.50	4.35	8.00
	Oestrone	0.65	0.80	2.55	6.05	5.20	4.70	8.10	6.15
	Oestradiol	-	0.10	0.35	1.20	0.90	1.10	1.65	1.70
C.T.	Oestriol	0.45	1.00	1.65	1.10	2.70	2.30	7.10	8.00
	Oestrone	0.30	0.50	1.20	2.35	2.15	2.05	2.85	5.15
	Oestradiol	nil	0.10	0.30	0.60	0.50	0.80	0.80	0.80

G.W.	Oestriol	nil	0.65	1.05	2.00	1.55	1.30	2.65	5.30
	Oestrone	0.50	0.75	0.55	1.15	2.20	1.75	3.95	4.10
	Oestradiol	nil	0.20	0.10	0.25	0.70	0.70	0.90	0.70
M.W.	Oestriol	0.15	0.25	1.40	1.80	1.25	2.40	2.30	-
	Oestrone	0.15	0.30	1.00	1.20	1.70	2.05	2.55	-
	Oestradiol	0.05	0.05	0.05	0.40	0.60	0.50	0.90	-
R.F.	Oestriol	1.4	1.10	1.00	1.50	1.60	5.55	4.90	8.50
	Oestrone	nil	0.25	0.20	0.60	0.45	1.10	1.70	1.80
	Oestradiol	nil	0.20	0.30	0.35	0.20	0.45	1.00	0.50
M.R.	Oestriol	0.30	1.05	1.30	1.75	-	3.85	3.80	4.50
	Oestrone	0.60	0.60	2.20	3.10	4.15	1.55	1.45	0.80
	Oestradiol	0.10	0.10	0.10	0.80	1.00	1.00	1.20	1.00
Mean±SD	Oestriol	0.20±0.16	0.75±0.46	1.63±0.56	2.43±0.94	2.79±0.91	3.26±2.00	5.99±3.56	9.65±5.13
	Oestrone	0.39±0.36	0.66±0.46	1.63±1.12	2.80±1.83	3.73±2.06	3.92±2.48	4.53±2.58	5.44±2.98
	Oestradiol	0.05	o.10±0.o7	0.27±0.20	0.53±0.34	0.65±0.34	0.90±0.20	0.89±0.41	1.19±0.41
	TOTAL	0.46±0.12	1.5 ±0.78	3.66±1.48	5.75±2.21	7.17±2.76	8.08±4.56	11.41±5.58	16.40±7.08

All results in µg/100 ml.

Patients R.F. and M.R. omitted from mean.

TABLE 29

Clinical Details of Patients delivered by Caesarean Section

Patient	Age	Parity	Duration	Baby wt. lb.	Baby wt. oz.	Placental wt lb.	Placental wt oz.	Indications for Caesarean Section
M.P.	27	3	$38^{6}/_{7}$	7	15	1	3	Type II placenta praevia
M.McC.	22	2	$40^{1}/_{7}$	6	15	1	7	Contracted pelvis, previous C/S.
E.A.	31	2 + 1	$39^{4}/_{7}$	9	2½	1	1	Elective; 2 previous C/S.
T.D.	29	2	$41^{6}/_{7}$	9	3½	1	2	Prolonged labour; failed pitocin drip.
O.P.	40	3 + 1	$41^{4}/_{7}$	6	12			Failed pitocin drip.
C.S.	26	0	$39^{6}/_{7}$	4	11½		12	Contracted pelvis; neonatal death at 2 weeks from congenital heart abnormalities
A.C.	39	0	$39^{1}/_{7}$	5	7		15	Elective; mild pre-eclampsia; contracted pelvis; elderly primigravida.
L.G.	28	1	$39^{2}/_{7}$	5	15		15	Contracted pelvis; previous C/S.
M.R.	30	1	$40^{6}/_{7}$	8	0½	1	2	Elective; previous C/S; contracted pelvis.
A.B.	33	1 + 2	42	9	6½	1	7	Previous C/S; terminal rise in blood pressure.
N.K.	21	1	$40^{2}/_{7}$	6	15	1	10	Elective; previous C/S; contracted pelvis.
J.C.	25	N.N.D. 1	$39^{6}/_{7}$	6 6	11 6½	1 1	4 3	Previous C/S; binovular twins.
A.I.			$37^{2}/_{7}$	5 5	5 6¼	2	2	Prolonged labour; contracted pelvis; uni-ovular twins.

TABLE 30

BLOOD OESTROGEN LEVELS IN

Patient	OESTRIOL				
	Liquor	Umb. vein	Umb. art.	Uterine vein	Arm vein
M.P.	52.0	22.5	26.0	5.45	4.25
M.McC.	60.0	32.0	* -	16.45	14.30
E.A.	71.3	46.7	50.0	9.70	7.85
T.D.	52.0	30.5	* -	* -	22.60
O.P.	34.0	43.0	47.5	7.50	7.25
C.S.	124.0	93.7	84.5	5.10	4.75
A.C.	56.7	34.0	31.5	7.10	3.60
L.G.	60.0	33.0	26.0	5.86	3.10
M.R.	* -	45.5	45.7	5.40	4.70
A.B.	89.0	47.0	46.7	8.50	3.90
N.K.	71.7	47.0	52.0	3.30	3.20
J.C.	120.0	106.0 91.0	91.3 98.0	6.20	6.00
A.I.	86.0	I 52.0 II 54.3	I 55.3 II 51.3	23.60	17.80
† Mean	73.10	55.10	54.30	8.70	6.73
± S.D.	±33.09	±25.52	±22.41	±5.76	±4.67

all values given as µg/100 ml.

† Mean values calculated from those results where values were available for both members of the pair of vessels to be compared.

TABLE 30

PATIENTS DELIVERED BY CAESAREAN SECTION

OESTRONE					OESTRADIOL				
Liquor	Umb. vein	Umb. art.	Uterine vein	Arm vein	Liquor	Umb. vein	Umb. art.	Uterine vein	Arm vein
0.40	0.50	0.60	2.30	1.80	0.25	0.40	0.40	1.10	0.60
0.30	0.20	*-	4.90	4.00	0.20	0.20	-	2.20	1.20
0.40	0.50	0.50	1.90	1.35	0.10	0.15	0.10	1.05	0.65
0.80	0.45	0.60	*-	1.90	0.20	*-	*-	*-	0.75
0.30	0.60	0.45	3.10	2.70	0.20	0.20	0.20	0.90	0.95
0.75	1.30	2.00	3.50	2.90	0.40	0.30	*-	0.60	0.60
0.40	0.40	0.30	1.10	0.80	0.40	0.10	0.10	1.80	0.80
0.40	0.30	0.40	1.20	1.35	0.20	0.10	*-	0.60	0.30
*-	0.60	0.80	0.60	0.40	*-	0.30	0.30	0.60	0.50
0.60	0.60	0.30	3.00	1.50	0.30	0.40	0.30	2.00	0.80
0.30	0.50	*-	1.60	1.20	*-	0.10	*-	1.40	1.00
0.50 I	0.60 I	0.40	1.10	1.20	0.40 I	0.60	0.50	0.80	0.95
II	0.60	0.60			II	0.60	0.60		
0.90 I	0.60 I	*-	6.00	4.70	0.10 I	0.30 I	*-	2.65	2.40
II	0.70 II	*-			II	0.40 II	*-		
0.50	0.59	0.45	2.53	1.99	0.24	0.34	0.31	1.31	0.895
±0.21	±0.25	±0.48	±1.65	±1.32	±0.13	±0.19	±0.18	±0.70	±0.54

* Volume of blood too small for accurate measurement of oestrone and oestradiol or sample not collected.

TABLE 31

BLOOD OESTROGEN LEVELS IN PATIENTS

Patient	Age	Parity	Duration at Delivery	Baby wt. lb. oz.	Placental wt. lb. oz.	Indications for Caesarean Section
J.G.	29	1	$41^{3}/_{7}$	9 10 F.	2 4	Previous C/S for foetal distress; post-mature.
B.N.	29	0	$41^{2}/_{7}$	7 8 M.	1 8	Contracted pelvis
J.D.	39	0	42	7 15½ M.	1 6	Elderly primigravida; mild pre-eclampsia; post-mature.
I.M.	30	2	$38^{3}/_{7}$	6 8½ F.	1 6	Previous C/S; contracted pelvis; Rh iso-immunisation.
S.S.	28	1+1	$38^{6}/_{7}$	7 14 M.	1 6	Previous C/S; contracted pelvis; cardiac.
C.C.	25	1	$39^{6}/_{7}$	7 1½ F.	1 8	Previous C/S; contracted pelvis.
M.R.	30	0	$41^{4}/_{7}$	7 8 M.	1 12	Infertility
M.A.	27	2	$39^{1}/_{7}$	7 4	1 6	2 previous C/S; contracted pelvis.
E.G.	25	0	$40^{5}/_{7}$	5 0 F.	1 3	Contracted pelvis; breech presentation.
A.T.	28	0	$39^{6}/_{7}$	7 9 F.	1 1	Infertility; breech presentation.

TABLE 31

UNDERGOING ELECTIVE CAESAREAN SECTION

OESTRIOL		OESTRONE		OESTRADIOL	
Day I	Day II	Day I	Day II	Day I	Day II
19.40	15.90	6.75	8.20	1.40	1.80
3.20	6.95	1.40	1.35	0.60	0.65
7.80	5.65	0.65	0.40	0.65	0.65
5.10	6.15	1.10	1.10	0.55	0.70
8.80	5.80	3.95	2.55	0.90	0.95
6.70	6.65	0.65	0.85	0.70	1.05
8.85	8.60	2.05	2.80	1.10	0.85
3.80	3.55	0.85	0.90	0.55	0.50
5.63	5.40	3.01	3.00	0.95	0.70
9.70	9.40	3.45	1.90	0.90	0.90
Mean ± S.D. 7.90±4.60	7.31±3.36	2.39±1.95	2.31±2.26	0.83±0.29	0.88±0.36

All results expressed as µg/100 ml. blood.

TABLE 32.

OESTROGEN LEVELS IN PATIENTS

Patient	Parity	L.M.P.	Duration at Delivery	Date of assay	Duration at time of assay	Oestriol	Oestrone	Oestradiol	TOTAL
						(µg/100 ml)			
J.A.	1	17. 5.58	36 4/7	6. 1.59	33 3/7	3.80	0.90	1.00	5.70
				22. 1.59	35 3/7	1.45	0.10	-	-
F.T.	1	3. 7.58	41 1/7	17. 4.59	41 1/7	3.90	1.90	0.65	6.45
* M.W.	1	27. 8.58	31 3/7	3. 4.59	31 2/7	3.45	1.15	0.45	5.05
A.H.	1	15. 6.58	39 5/7	20. 3.59	39 5/7	3.90	2.40	0.95	7.25
J.M.	2 (0+1)	20. 6.58	38 2/7	16. 3.59	38 1/7	7.10	1.30	0.80	9.20
* D.C.	1	?. 6.58	?36	6. 3.59	?36	4.20	3.40	1.10	8.70
E.C.	1	23. 5.58	39 1/7	21. 2.59	39 1/7	18.5	2.55	0.90	21.95
H.O.	1	27. 8.58	39 3/7	29. 5.59	39 3/7	8.90	7.50	1.50	17.90
M.Wh.	1	21. 9.58	37 5/7	5. 6.59	36 5/7	25.00	4.40	1.40	30.80
				8. 6.59	37 1/7	22.30	2.30	0.30	24.90
C.R.	1	28. 8.58	40 1/7	4. 6.59	40	6.80	2.80	1.00	10.60
G.B.	1	8. 8.58	40 1/7	15. 5.59	40 1/7	3.85	2.25	0.70	6.80
R.V.	1	18.10.58	35 2/7	18. 6.59	34 5/7	3.40	3.05	1.35	7.80
* J.W.	1	29. 3.59	33 6/7	20.11.59	33 5/7	4.60	1.20	1.10	6.90
A.W.	1	3. 3.59	37 6/7	19.11.59	37 2/7	10.30	3.50	1.20	15.00
† M.M^{c}A.	1	20.10.58	35 5/7	15. 5.59	30	1.35	0.35	0.10	2.80
† C.W.	1	3. 5.59	29	19.11.59	29	0.80	0.70	0.70	2.20
† J.B.	2 (0+1)	24. 8.58	34 1/7	19. 4.59	34	2.45	0.25	0.20	2.90
† (S.D.)	1	16. 3.59	35 2/7	5.11.59	33 3/7	1.20	0.60	0.20	2.00
				6.11.59	33 4/7	0.90	0.60	0.15	1.65
				7.11.59	33 5/7	0.55	0.45	0.40	1.40
† (M.M.)	1	30. 3.58	40	18.12.60	37	0.50	0.35	0.40	1.25

* M.W. Neonatal death - presence of pulmonary hyaline membrane
D.C. " " - pulmonary oedema
J.W. " " -

TABLE 32.

WITH PRE-ECLAMPTIC TOXAEMIA

Baby weight lb.	oz.	Placental weight lb.	oz.	Maximum blood pressure	Albumin-uria g/l	Clinical details Severity	Onset	Treated with
4	13½	1	1½	180/120	4.0	moderate → severe	< 31 weeks	Avertin
8	2½	1	9	190/115	13.0	severe	?early April	Avertin
2	13½		14	190/120	trace	severe	28 weeks, neonatal death (3 days)	
5	11		15	200/140	+	severe	35 weeks	Avertin
7	12¼	1	5	190/120	0.5	severe	29 weeks	Avertin
5	2¾	1	1	180/120	6.0	severe	?34 weeks, neonatal death (6 days)	Avertin
7	2	1	6	200/120	?7.0	fulminating	<2 weeks, 37 weeks	Avertin
7	10	1	7	160/100	+	moderate	? 38 weeks	
6	7	1	3½	170/110	3.0	moderate → severe;	? 37 weeks	Avertin
9	4	1	13	180/120	3.0	moderate	? 36 weeks	
6	5½	1	8	190/130	4.0	severe	37 weeks	Avertin
5	7½	1	1	200/100	3.0	severe	?32 weeks	
4	9	1	2	190/100	12.0	fulminating; neonatal death (3½ hours)		
6	10	1	12	160/110	zero		?34 weeks	
4	5		9	160/120	8.75		29 weeks, stillbirth	
1	6		6	178/130	10.0	severe	stillbirth	Avertin
2	11		6	200/115	18.09	eclampsia;	stillbirth	
3	3		7	160/100	zero	moderate;	stillbirth	Serpasil
4	7		14			moderate;	28 weeks, stillbirth	Serpasil Saluric

† M.M^C A. Foetal heart not heard in labour
C.W. " " " " 36 hours prior to labour (this was only the second time the patient was seen, the first occasion being in June, i.e. 5 months previously).
J.B. Foetal heart not heard 2 hours before delivery.
S.D. Foetal heart heard on 4.11.59, not on 7.11.59
M.M. Foetal heart not heard on 17.11.58.

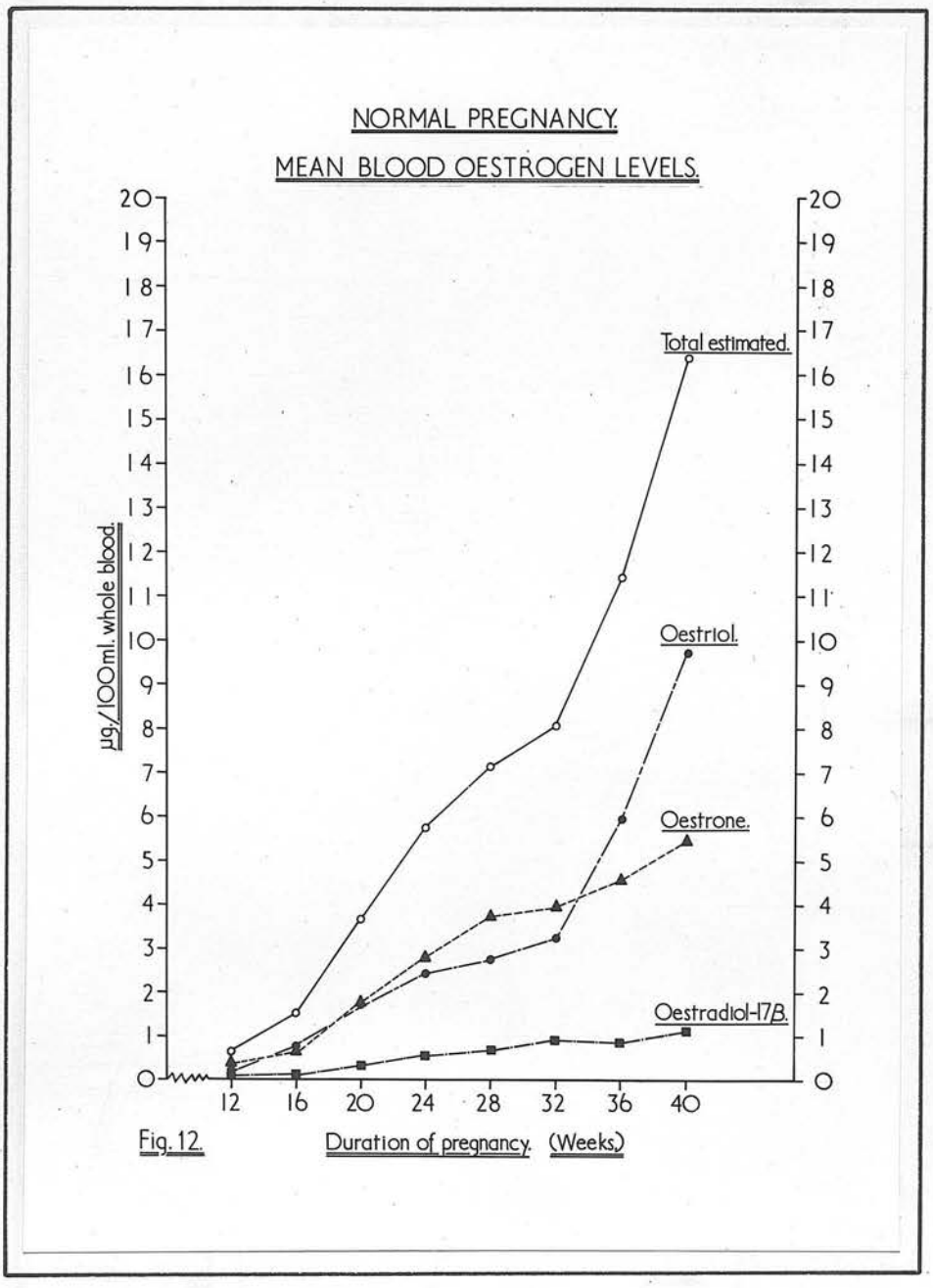
NORMAL PREGNANCY.
MEAN BLOOD OESTROGEN LEVELS.
µg./100ml. whole blood.
Total estimated.
Oestriol.
Oestrone.
Oestradiol-17β.
Duration of pregnancy. (Weeks.)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
12
16
20
24
28
32
36
40

Fig. 12.

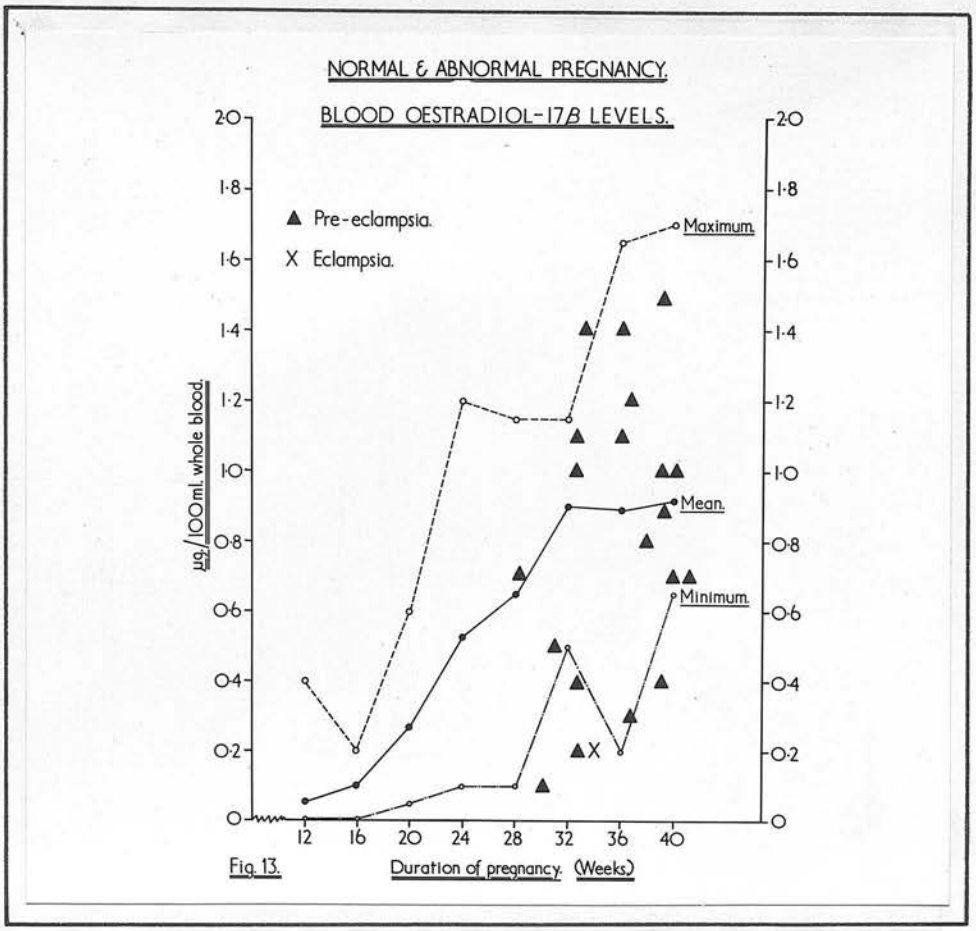
NORMAL & ABNORMAL PREGNANCY.
BLOOD OESTRADIOL-17β LEVELS.
▲ Pre-eclampsia.
X Eclampsia.
Maximum.
Mean.
Minimum.
µg./100 ml. whole blood.
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
12
16
20
24
28
32
36
40
Duration of pregnancy. (Weeks.)

Fig. 13.

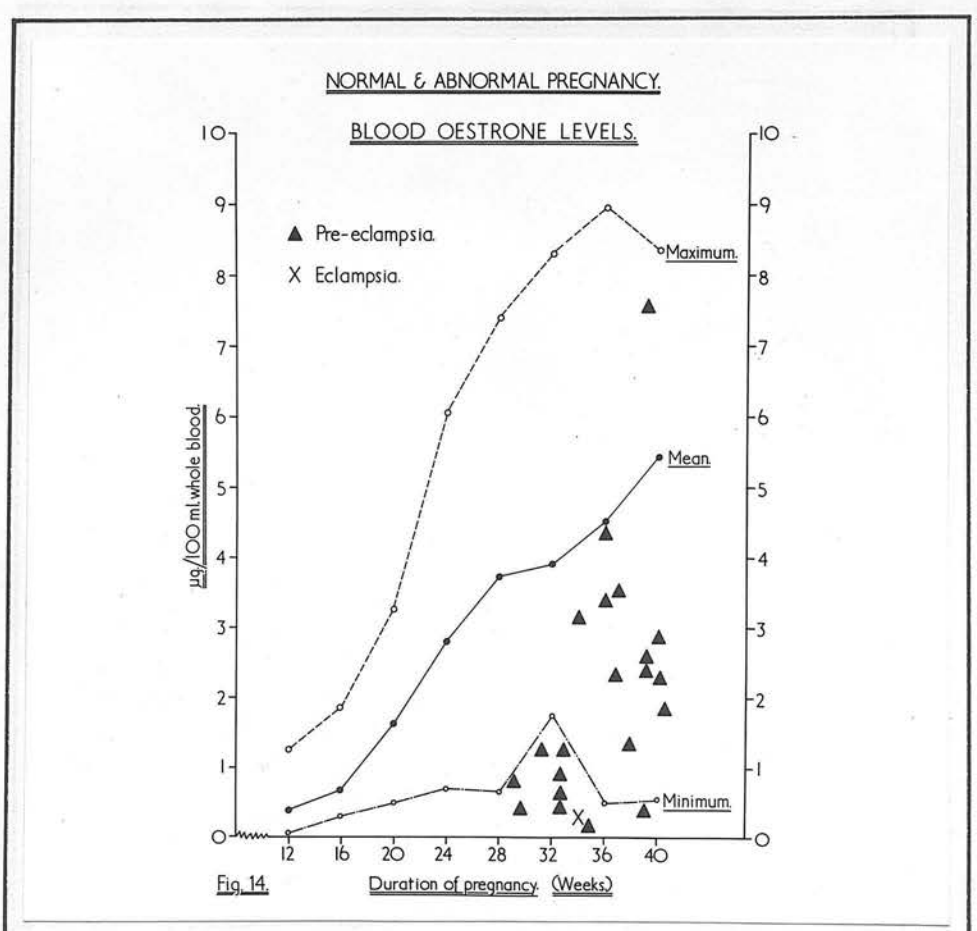
NORMAL & ABNORMAL PREGNANCY.
BLOOD OESTRONE LEVELS.
▲ Pre-eclampsia.
X Eclampsia.
Maximum.
Mean.
Minimum.
µg./100 ml. whole blood.
10
9
8
7
6
5
4
3
2
1
0
12
16
20
24
28
32
36
40
Duration of pregnancy. (Weeks.)

Fig. 14.

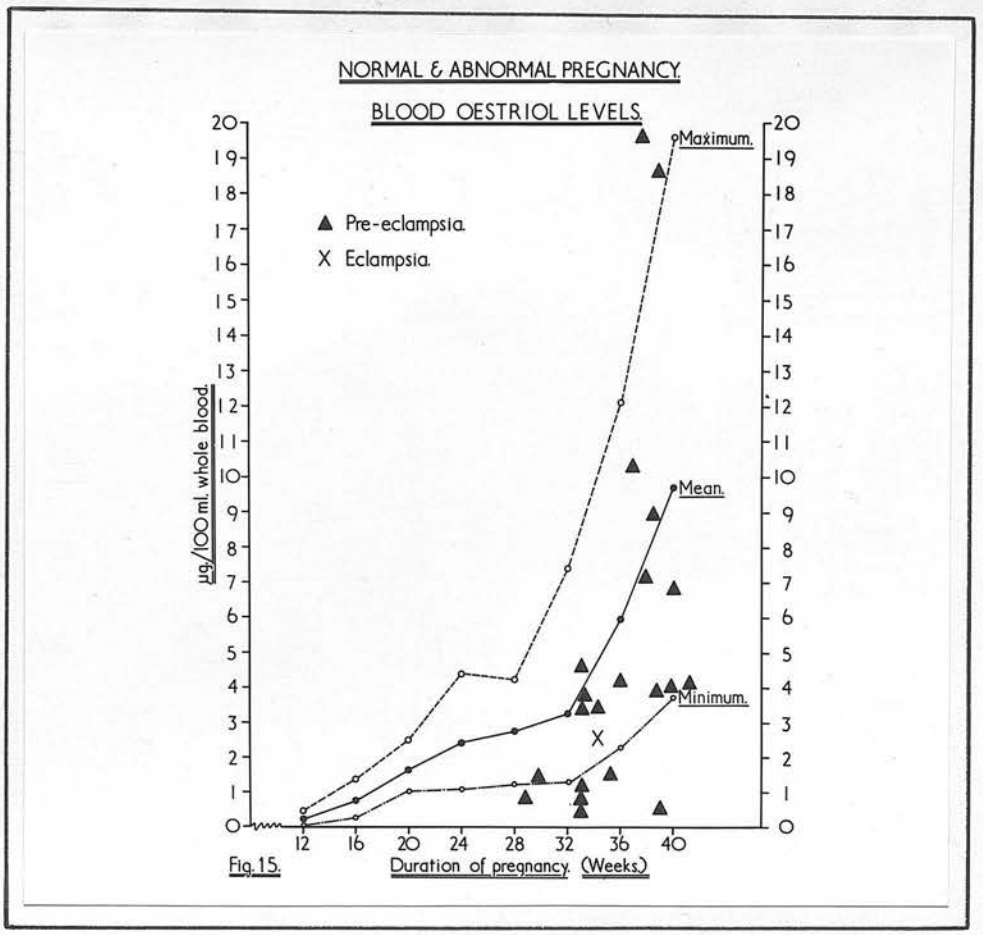
NORMAL & ABNORMAL PREGNANCY.
BLOOD OESTRIOL LEVELS.
Maximum.
Mean.
Minimum.
▲ Pre-eclampsia.
X Eclampsia.
µg/100 ml. whole blood.
Duration of pregnancy. (Weeks.)

Fig. 15.

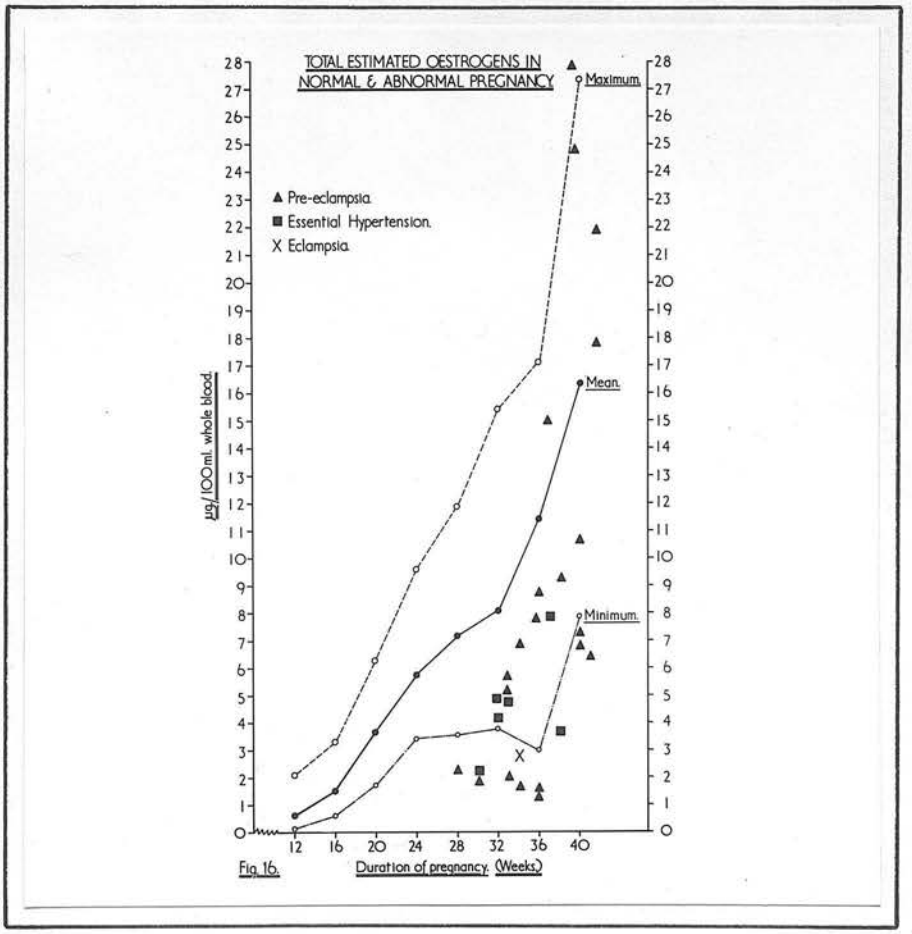
TOTAL ESTIMATED OESTROGENS IN
NORMAL & ABNORMAL PREGNANCY
Maximum.
Mean.
Minimum.
▲ Pre-eclampsia.
■ Essential Hypertension.
X Eclampsia.
µg/100 ml. whole blood.
Duration of pregnancy. (Weeks.)

Fig. 16.

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* deceased.

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PUBLICATIONS.

SOME CLINICAL APPLICATIONS OF OESTROGEN ASSAY*

BY

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GYNAECOLOGICAL CONSIDERATIONS

MUCH of our past knowledge of the role of the oestrogens has been obtained by indirect means. For instance, the administration of oestrogens to a patient with a senile vaginitis caused thickening of the vaginal skin and consequent amelioration of the condition. Their use in patients suffering from secondary amenorrhoea led to endometrial stimulation and consequent bleeding. These were rather obvious examples of oestrogen lack. In cystic glandular hyperplasia it was certain that there was a state of excess oestrogen production and the degree of myometrial and endometrial hyperplasia was good evidence of this. Certain hormone producing tumours such as a granulosal cell tumour might have a rejuvenating effect when they occurred in elderly women. In all these examples the amount of oestrogen present could be estimated in an approximate way by endometrial biopsy or vaginal smear or more exactly by biological quantitative estimations. Oestrogens have been thought to be implicated in such conditions as uterine fibroids, carcinoma of the cervix and of the endometrium, endometriosis and the like, but in these conditions any real evidence of abnormal oestrogen production has been lacking. With the introduction of the chemical quantitative methods it has become possible to measure the urinary oestrogen excretion in a wide variety of gynaecological conditions and we now have a reasonable idea of what is happening in many of them.

I.—THE POST-MENOPAUSAL PATIENT

The total mean urinary oestrogen excretion in a post-menopausal patient is of the order of 5·8 μg. per 24 hours with a range of 3·1 μg. to 8·1 μg. It is reasonably certain that the source of this oestrogen is the adrenal glands for their removal leads to a fall in oestrogen excretion to very low levels.

Post-menopausal Bleeding

During the past few years we have had the opportunity of measuring the oestrogen output in a variety of conditions giving rise to post-menopausal bleeding and the following are the sort of results we obtained.

(1) In some cases the source of the post-menopausal bleeding was a cervical or endometrial polyp and in such patients there was no alteration in the normal post-menopausal levels of excretion. Similarly, patients who have a post-

* Presented by Professor Kellar.

menopausal haemorrhage, and in whom an atrophic endometrium is found or in whom no curettings are obtained, fall into this group.

(2) *Ovarian tumours.* We have now had the opportunity of studying several patients presenting with post-menopausal bleeding and in whom a *simple ovarian tumour* has been discovered and removed. In one such patient a daily excretion of 45 μg. total oestrogen was found, falling to post-menopausal levels after the removal of a pseudomucinous ovarian cyst and hysterectomy. Extensive histological examination of the tumour failed to reveal any granulosal elements although the endometrium was typical of cystic glandular hyperplasia. In these cases it must be assumed that the stroma supporting the epithelium is functioning as an oestrogen source. We have also noted this phenomenon in *malignant ovarian tumours.* Unfortunately we have not had the opportunity of carrying out many observations on post-menopausal patients with oestrogenic ovarian tumours. One elderly patient with post-menopausal bleeding was found to have a cystic glandular hyperplasia of the endometrium and an ovarian tumour. She was excreting over 30 μg. of oestrogen daily before the removal of what proved to be a granulosal cell tumour. It will be noted, therefore, that ovarian tumours, benign, malignant or hormone-producing, can give rise to levels of oestrogen sufficient to cause endometrial proliferation and bleeding after the menopause.

Carcinoma of the Cervix

In 6 patients in our original series the cause of the post-menopausal bleeding was a carcinoma of the cervix. The mean total oestrogen excretion was 5·6 μg. daily, thus falling into the normal post-menopausal range.

Carcinoma of the Endometrium

In 5 cases the cause of the post-menopausal bleeding was found to be an adenocarcinoma of the endometrium, and again the mean total oestrogen excretion level of 5·5 μg. was that of the normal post-menopause.

In this very small series of 11 patients with carcinoma of cervix and endometrium there is no evidence of any abnormal excretion of oestrogens. This group is well worthy, however, of more extensive study and we hope we may hear of other observations this afternoon.

II.—The Amenorrhoeas

These patients form a heterogeneous group and our results of oestrogen assay can be briefly summarized as follows:

Primary Amenorrhoea. Congenital absence of the uterus and vagina. In 3 such patients it was found that there was evidence of cyclical activity of the ovaries, thus indicating that this activity can occur in the absence of the target organs.

Secondary Amenorrhoea. In one group the oestrogen excretion is low and within the post-menopausal level. The endometrium, if any can be obtained, is in a resting phase. In the majority of such patients spontaneous return of ovarian activity is unlikely and many of these patients would appear to have a premature menopause.

In the second group the oestrogen output is within proliferative levels and the endometrium shows varying degrees of activity. Most of the patients we have studied have shown a spontaneous return of menstrual function after a variable period of amenorrhoea. This grouping of patients suffering from amenorrhoea into those with a post-menopausal level of oestrogen excretion and a poor prognosis and those with a proliferative level and a better prognosis is probably reasonably accurate but it is not always correct, and we have had one patient with a low menopausal level who later had a spontaneous return of menstrual function and became pregnant.

It is important that the oestrogen excretion levels should be studied over a reasonable period of time and reliance should not be placed on a few or isolated observations. We are at present studying patients suffering from amenorrhoea with a view to analyzing the precise oestrogen/gonadotrophin relationships.

III.—Dysfunctional Uterine Bleeding

The group of patients suffering from dysfunctional uterine bleeding is a very difficult one for study as the patient requires to have

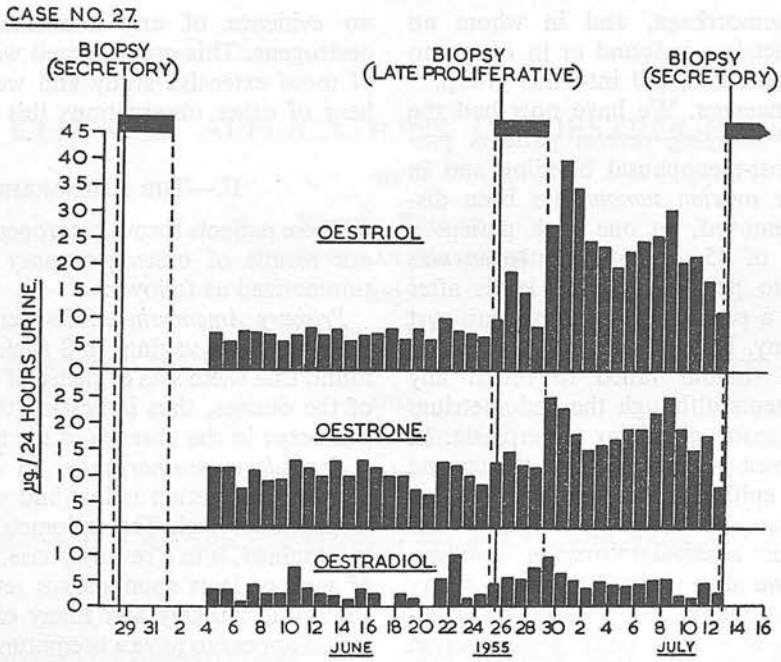

FIG. 1

Anovulatory menstruation (Brown *et al.*, 1959).

oestrogen assays over a prolonged period of time if any real knowledge is to be obtained.

Anovulatory Cycles

This is perhaps the simplest form of dysfunctional uterine bleeding and we have been fortunate in being able to study some 11 anovulatory cycles. The anovulatory cycle is characterized by the relative constancy of the total daily oestrogen excretion which varies between the range 13 μg.–30 μg. Figure 1 shows a very typical example of an anovulatory cycle with a steady oestrogen output of 20 μg. daily and followed by an ovulatory cycle.

Cystic Glandular Hyperplasia

It is not easy to obtain suitable patients suffering from cystic glandular hyperplasia for adequate study as so often some form of definitive treatment has to be given.

In the relatively few cases that we were able to study for many weeks and also in those in whom only short periods of investigation were possible, it was found that, in general, cystic glandular hyperplasia of the endometrium is associated with daily total oestrogen excretion rates of the order of 30 μg. In some cases those excretion levels were maintained for several weeks. Daily rates of over 100 μg. have been recorded.

It was noted that in some cases of cystic glandular hyperplasia the daily excretion rates, instead of remaining relatively constant, might show broadly fluctuating levels and the patient might have what were to her relatively normal periods.

Finally, it became clear that uterine bleeding in this condition might be associated with rising, falling or relatively constant levels of oestrogen excretion.

The following figures which were published in the *Journal* earlier this year depict the oestrogen excretion levels in cystic glandular hyperplasia (Brown *et al.*, 1959) (Figs. 2, 3, 4 and 5).

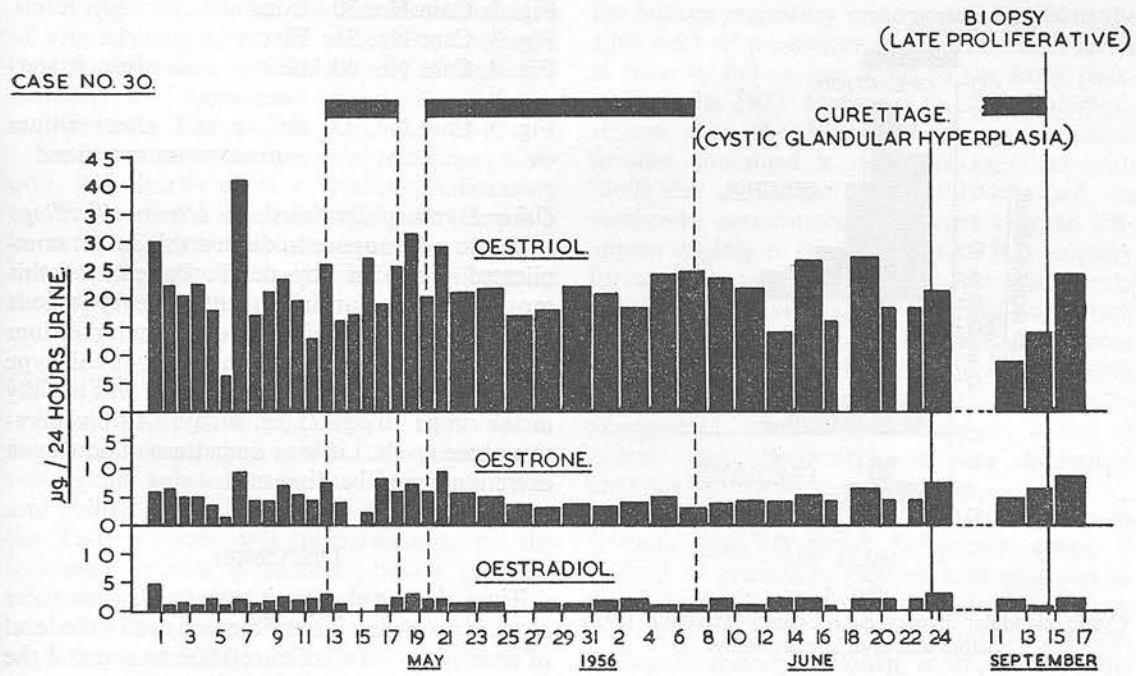

FIG. 2

Cystic glandular hyperplasia. Constant oestrogen excretion levels.

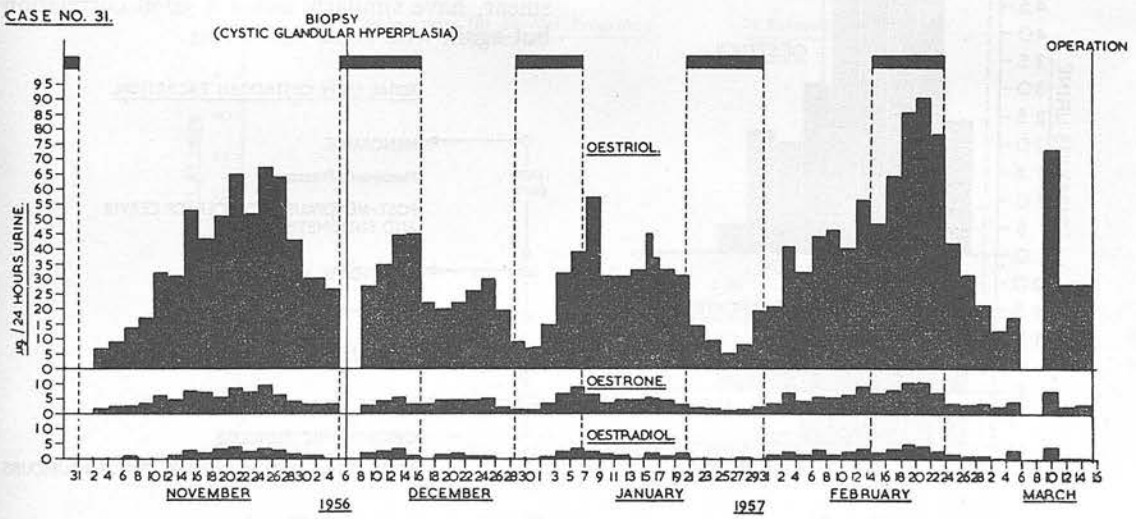

FIG. 3

Cystic glandular hyperplasia. Fluctuating excretion levels.

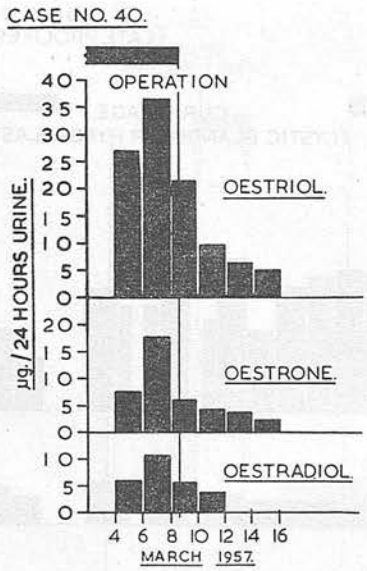

FIG. 4
Cystic glandular hyperplasia. Urinary excretion levels before and after hysterectomy.

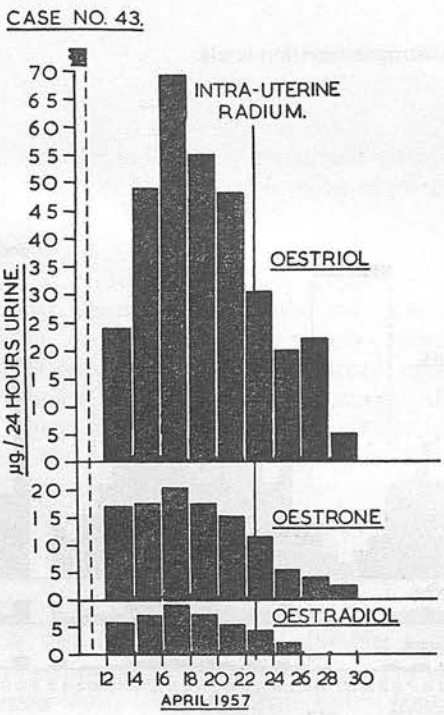

FIG. 5
Cystic glandular hyperplasia. Urinary excretion levels before and after intra-uterine radium.

Fig. 2, Case No. 30. Constant oestrogen levels.
Fig. 3, Case No. 31. Fluctuating levels.
Fig. 4, Case No. 40. Before and after hysterectomy.
Fig. 5, Case No. 43. Before and after radium menopause insertion.

Other Forms of Dysfunctional Uterine Bleeding

We do not propose to discuss this rather complicated group in any detail. Patients of this group had irregular, infrequent or heavy periods and histological examination of the endometrium showed a proliferative endometrium. In this type of case the total oestrogen excretion was usually in the range 10 µg.–25 µg. a day, i.e., proliferative phase levels. Little or no pattern of oestrogen excretion could be discerned.

DISCUSSION

Time does not permit me to discuss these results in any detail but Figure 6 shows the level of oestrogen excretion in relation to some of the gynaecological disorders studied.

In general, there was a good correlation between the oestrogen excretion levels and the state of the endometrium although there were some exceptions. Puttarajurs and Taylor (1959), in their excellent study on the relationship between oestrogen excretion and the vaginal smear, have similarly noted a good correlation but again with some exceptions.

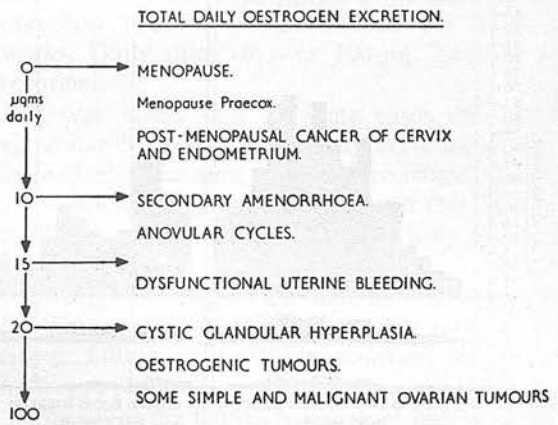

FIG. 6
Urinary oestrogen excretion levels in various gynaecological disorders.

It may be argued that the chemical estimation of urinary oestrogens will seldom be necessary for the clinician and that the study of the endometrium or of the vaginal smear will satisfy his requirements. This may well be the case.

Simplification of the chemical technique, however, will shortly allow a "routine" laboratory to offer oestrogen determinations, and there will be many patients in whom it will be useful to know the oestrogen excretion in diagnosis and in therapeutic control.

Obstetrical Considerations

Even before the first world war certain German authors had noted the presence of oestrogenic substances in the human placenta and Fellner (1912) is generally credited as being the first to note this phenomenon. In the following decades it became obvious that the placenta was indeed a rich source of oestrogenic materials and by 1930 pure oestriol had been identified in extracts of the human placenta. In 1927 Aschheim and Zondek first noted the presence of large quantities of oestrogenic substances in human pregnancy urine and in the following year demonstrated that oestrogens in the human pregnancy urine rose from about the 12th week of pregnancy to relatively high levels at term to fall to low levels in the early puerperium. In 1927 Margaret G. Smith demonstrated that the blood of pregnant human females contained a substance identical with "follicular hormone", and this reached its maximum concentration at term only to disappear rapidly in the puerperium. It is salutary for us, then, to remember that over thirty years ago it was known that the placenta was a rich source of oestrogens and that these substances appeared in the blood and urine in increasing quantities until term when they promptly disappeared. All this painstaking work had of course been dependent on the biological methods of estimating oestrogens.

In 1935 Guy Marrian and his co-workers in Toronto had developed a purely chemical method of estimating oestriol and oestrone in the urine and in that year published their now classical paper on the "Excretion of Oestrin during Pregnancy" (Cohen *et al.*, 1935). This was, we are told by our chemical colleagues, the first time that a steroid substance was accurately measured in a body fluid by a purely chemical method. At this time Marrian believed that

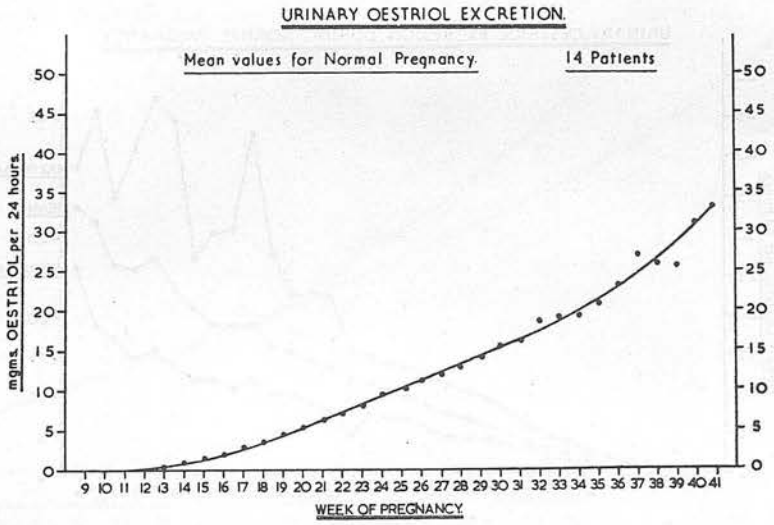

FIG. 7

Mean urinary oestriol excretion during normal pregnancy. Based on 309 observations on 14 normal pregnancies.

oestriol was excreted in a conjugated form until shortly before term when most of it was excreted in a "free" form. Years later he was to show that this was due to the fact that the urines supplied to him from late pregnancy patients by his obstetrical colleagues were contaminated by other body fluids especially cervical mucus—a rich source of the enzyme glucuronidase, and, in fact, if catheter specimens were used it was found that oestriol was largely excreted in the conjugated form right up until delivery. If we add his conjugated and free figures for oestriol together we obtain the graph (Fig. 9) which shows the excretion of oestriol throughout pregnancy and one which, we shall see shortly, is remarkably similar to those which have been obtained in recent years by the use of improved chemical methods.

At this point we would like to recall to you that in 1934 another classical paper had been published in the *American Journal of Physiology* by George and Olive Smith. This paper was entitled "Excessive gonad-stimulating hormone and subnormal amounts of estriol in the toxemias of late pregnancy" and was one of the first of a long series of distinguished communications on this subject that these authors were to make. We are sure that many present will scarcely realize that this fundamental contribution was made almost a quarter of a century ago.

Excretion of Oestriol in Normal Pregnancy Urine (Edinburgh Studies)

Brown (1956) published the details of his method of measuring oestradiol, oestrone and oestriol in the urine and in the following year was able to demonstrate the application of his method to the urinary oestrogen excretion in normal pregnancy. He showed that the three oestrogens increased from the luteal phase of the cycle of conception until term when they fell rapidly. Oestriol excretion increased a thousandfold and oestrone and oestradiol thirtyfold from the 10th week.

Since 1956 we have been able to add a further ten normal pregnancies to his series and the following graph (Fig. 8), which we can refer to as the "Edinburgh oestriol excretion curve", is based on three hundred and nine observations on 14 normal pregnancies. As far as possible, weekly 24-hour urinary collections were obtained from the 9th to the 41st week of pregnancy. Figure 7 shows the mean oestriol curve

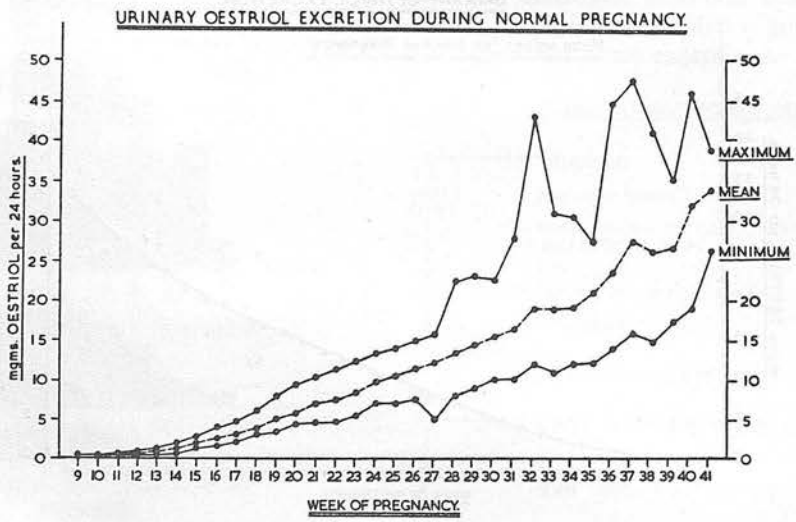

FIG. 8

Urinary oestriol excretion during normal pregnancy. Shows maximum, mean and minimum at each week of pregnancy in 14 normal pregnancies.

throughout pregnancy and it will be seen that there is a reasonably smooth rise in oestriol excretion from the early weeks until 40 weeks when approximately 31·5 mg. of oestriol are being excreted daily. When we look at Figure 8 on which the maximum, mean and minimum amounts of oestriol excreted each week are plotted, we see that there is a wide scatter of results which naturally becomes more obvious as we enter the third trimester of pregnancy. It will be clear that it will be only in the exceptional case that a single reading can have much significance. A similar wide scatter of results is noted in pregnanediol excretion in normal pregnancy. The significance is not clear as it is neither due to errors in urine collection nor to mistakes in chemical methodology.

Lenters in 1958 reported the results of his studies on oestriol excretion in normal and abnormal pregnancy, and we are all delighted that this afternoon Professor ten Berge is here to tell us something of this work. The method that Lenters used to measure oestriol was different from Brown's, depending on fluorimetry rather than on colorimetry. Figure 9 shows the Edinburgh and the Lenters oestriol curves, and it will be noted that although the two curves end at a very similar point at the 40th week of pregnancy, there is a discrepancy of several milligrams during several of the rather vital weeks of the third trimester. The chemists will have to tell us the reasons for this but it will be clear that we cannot directly compare results obtained by Brown's method with those of Lenters.

For historical interest we have included the Marrian curve of 1935 (Fig. 9). The oestriol levels are much lower throughout pregnancy but the curve makes a brave spurt towards term and is not much lower than the more recent figures.

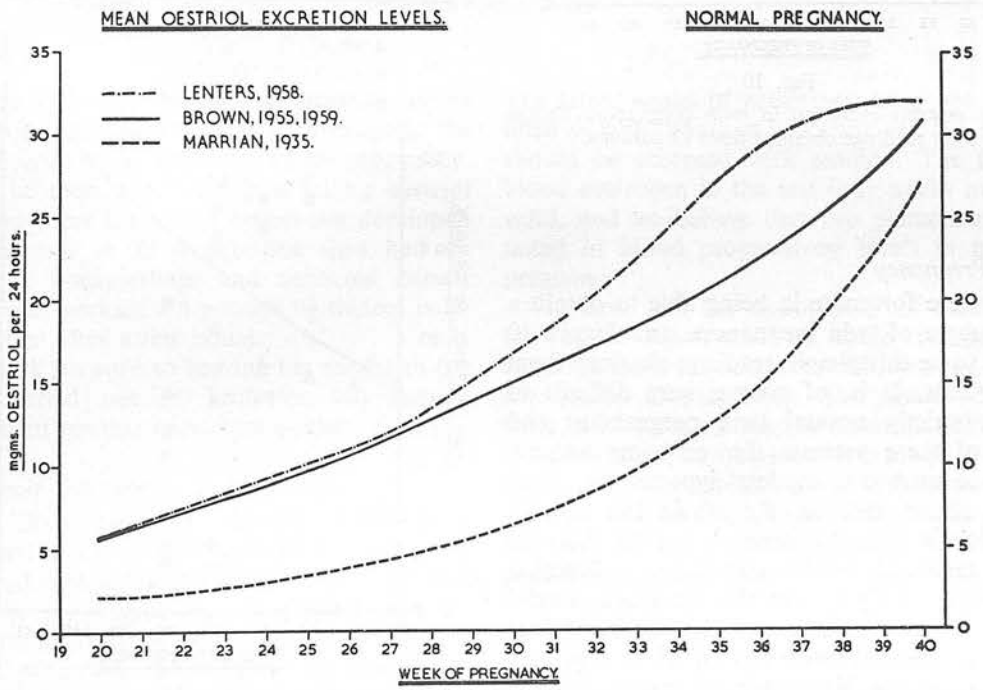

FIG. 9

Mean urinary oestriol excretion levels obtained by Lenters, Brown and Marrian.

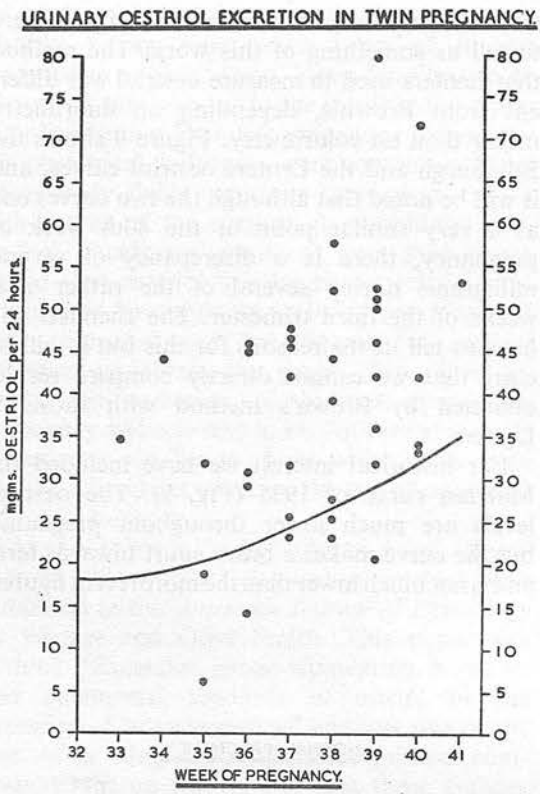

FIG. 10

Urinary oestriol excretion in twin pregnancy. Thirty-eight readings obtained from 12 patients.

Twin Pregnancy

We were fortunate in being able to obtain a fair sample of twin pregnancies and Figure 10 shows some thirty-eight readings obtained from 12 patients. It is, of course, very difficult to obtain strictly normal twin pregnancies and some of these patients showed some oedema and one or two a modest hypertension. This graph shows that on the whole the oestriol excretion in twin pregnancy is greater than the single series. If oestriol excretion is a function of placental weight, then this increased oestriol excretion could have been expected. The highest recorded oestriol excretion of 79·6 mg. was obtained at the 39th week of pregnancy in a binovular twin pregnancy delivered at the 40th week, the placentae weighing 3 pounds. It is doubtful, however, whether these higher figures are statistically significant.

Oestrogen Excretion in Pregnancy Toxaemia

The clinician still awaits some means of estimating placental function preferably by some fairly simple chemical method such as the measurement of a steroid metabolite. That there may be a reduction in oestriol excretion in certain cases of hypertensive toxaemia is undoubtedly true. We have not ourselves made any special study of this problem but in 12 patients with pre-eclampsia, who were under study for other purposes, we obtained the following results (Fig. 11). A fairly wide scatter of oestriol excretion rates is noted. Three patients who had excretion rates of less than 10 mg. daily after the 38th week, were delivered of babies under 5½ pounds.

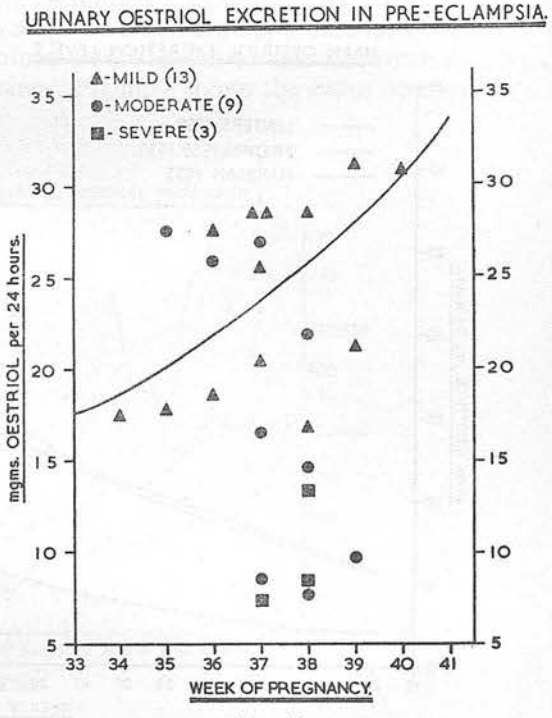

FIG. 11

Urinary oestriol excretion. Values obtained in 12 patients with varying degrees of pre-eclampsia.

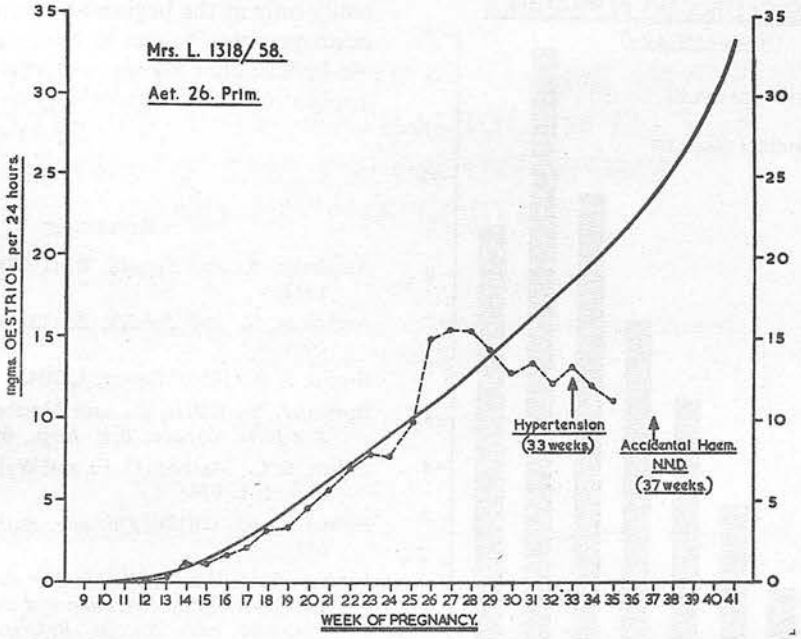

FIG. 12

Urinary oestriol excretion levels in a patient who developed pre-eclampsia and accidental haemorrhage.

Figure 12 shows the oestriol excretion curve of a patient who was originally included in the normal series at the beginning of her pregnancy. It will be seen that there is a falling oestriol excretion some little time before she developed pre-eclampsia at 33 weeks. She then had an accidental haemorrhage and delivered herself at the 37th week of a 5 pounds 9¼ ounces baby which died after a few hours.

We look forward to hearing the results of the work carried out in Professor ten Berge's department on this important subject.

The Blood Oestrogens in Pregnancy

Miss Roy and Dr. Brown (1959) have developed a colorimetric method for estimating the blood oestrogens (Oestradiol, Oestrone and Oestriol) in pregnancy. At present we are following a group of some 15 women through normal pregnancy and Figure 13 shows our results to date. There is a wide variation in the values of total oestrogens obtained at each month but the mean makes steady progress. The latter weeks of pregnancy have had to be filled in with "random" patients and the results should be accepted with caution. The fall in blood oestrogen in the last four weeks may be valid, and we believe that this phenomenon is noted in blood progesterone levels in human pregnancy.

We have had the opportunity of measuring the blood oestrogens in some 16 patients suffering from pre-eclampsia between the 30th–41st weeks of pregnancy. The results show a wide variation but it may be noted that although most of the values obtained were lower than the normal yet the highest blood oestrogen value we have obtained in any pregnant woman was 30·5 μg. per 100 ml. from a patient with severe pre-eclampsia with a normal urinary output.

CONCLUSIONS

Most of our remarks this afternoon have been of a factual nature and in the short time at our disposal we have avoided theorizing. We are

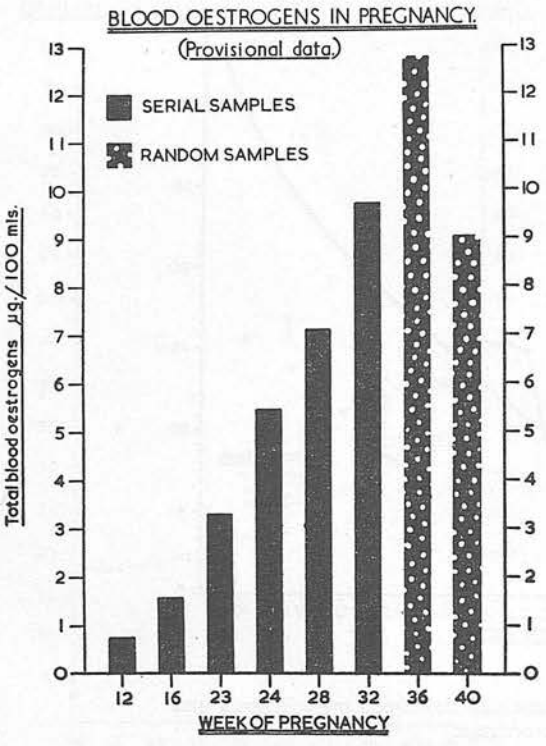

FIG. 13

Blood oestrogens (Oestradiol, Oestrone and Oestriol) in pregnancy. Serial follow-up to the 32nd week obtained from 15 normal pregnant women. Values at 36th and 40th weeks obtained from random samples.

really only at the beginning of our knowledge of oestrogen metabolism in health and disease, yet we believe that steady progress in the clinical application of our knowledge is being made.

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A METHOD FOR THE ESTIMATION OF OESTRIOL, OESTRONE AND OESTRADIOL-17β IN THE BLOOD OF THE PREGNANT WOMAN AND OF THE FOETUS

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SUMMARY

A chemical method for the estimation of oestriol, oestrone and oestradiol-17β in the blood of the pregnant woman and of the foetus is described. It is based on a method previously developed for urine and involves acid hydrolysis of whole blood, extraction of the phenolic fraction, methylation, chromatography on alumina columns and colorimetric measurement using a micro-modification of the Kober reaction. Kober-chromogenic impurities in the final extracts are derived from the procedure, not from the blood, and are eliminated by the spectrophotometric correction of Allen. The accuracy, precision, sensitivity and specificity of the method have been determined.

INTRODUCTION

Several chemical methods for measuring oestrogens in human plasma or blood have been reported (Veldhuis, 1953; Varangot, Seeman & Cedard, 1955; Preedy & Aitken, 1957). These methods have usually been applied to the measurement of oestriol, oestrone and oestradiol-17β (hereafter referred to as 'oestradiol') in the blood of both pregnant and non-pregnant individuals, usually with positive results. In all of these methods, the oestrogens are measured fluorimetrically by the fluorescence which develops when they are heated with sulphuric acid. Many of the impurities in the oestrogen fractions interfere in this measurement, either by fluorescing themselves or by partially quenching the fluorescence of the oestrogens. Varangot *et al.* (1955) ignored this interference and obtained oestrogen values which were some hundred times greater than those obtained by more specific methods. Veldhuis (1953) introduced corrections for this interference and obtained more acceptable figures, but so many assumptions were involved that proof of reliability of the results was difficult. Preedy & Aitken (1957) derived the specificity of their results from following the behaviour of the fluorescent material during fractional gradient elution chromatography. This expedient involves the fluorimetric analysis of large numbers of fractions from the chromatograms, and although it gives excellent results, it is not suited to the assay of large numbers of blood samples.

The Kober reaction, used either for colorimetry or fluorimetry (Kober, 1931; Ittrich, 1958), is highly specific for many of the natural oestrogens, and convenient methods for measuring urinary oestriol, oestrone and oestradiol, based on this reaction (Brown, 1955; Bauld, 1956; Brown, Bulbrook & Greenwood, 1957*a*), have proved reliable (Gallagher, Kraychy, Fishman, Brown & Marrian, 1958). The method for blood described here is adapted from that developed by Brown (1955) (method A) for measuring urinary oestrogens. It employs a 'micro' modification of the Kober colour reaction and is sensitive enough for measuring oestriol, oestrone and oestradiol in 10 ml. samples of blood or plasma from women during the second and third trimesters of pregnancy and in cord blood at birth. The method is suitable for day-to-day routine use, four determinations being possible in $1\frac{1}{2}$ working days. Another modification of method A has already been described for measuring oestrogens in human placentae and in foetal tissues and blood (Diczfalusy & Lindkvist, 1956; Diczfalusy & Magnusson, 1958).

MATERIALS AND METHODS

Apparatus and reagents

The apparatus and reagents are generally the same as those described for method A (Brown, 1955). Chromatography is performed in 10 mm × 5 cm sintered glass filter tubes of porosity no. 3, and sealed on to a reservoir of approx. 25 ml. capacity. The oestrogen fractions are eluted into $12 \times 1\frac{1}{2}$ cm tubes, fitted at one end with a B14 socket for connexion to the evaporation apparatus and narrowed at the other for a length of 10 mm to a diameter of 10 mm. The Kober colour reaction is performed in this narrowed section of the tube.

Optical densities are measured in a Unicam S.P. 600 spectrophotometer using micro cells with a light path of 10 mm and a capacity of 0·3–0·5 ml.

Ethanol (absolute) is allowed to stand over *m*-phenylenediamine for approx. 1 week and is then thrice distilled, the second distillation being performed using a fractionating column.

Alumina 'for chromatography' (Savory and Moore Ltd, London) is deactivated with 5–10 % of water to the activity specified for method A.

Standard solutions of pure oestrogens for recovery experiments, and of pure oestrogen methyl ethers for calibration graphs, contain 1 μg/ml. in ethanol, and are stored in the refrigerator. They are prepared at intervals by diluting solutions containing 50–100 μg/ml. which are stable indefinitely.

Kober colour reagents containing quinol, sulphuric acid and water are prepared as described for method A, except that the quinol concentration used in the oestradiol reagent has been reduced to 1·5 % (w/v).

THE METHOD IN DETAIL

Four determinations (two in duplicate) are usually performed at one time. Slightly more than 20 ml. of blood are collected by venipuncture into tubes containing 50–60 mg sodium oxalate to prevent clotting. Laking or clotting reduces the precision of the assay. The blood is stored at 4° C and processed within 1 week of collection. When plasma is being processed, the blood (10 ml.) is centrifuged soon after collection

and the plasma is removed; the red blood cells are then suspended in isotonic saline (10 ml.), the suspension is centrifuged, and the supernatant is added to the plasma.

A flow sheet of the extraction procedure is given in the earlier description of method A (Brown, 1955).

Hydrolysis, extraction, separation of phenol fractions and methylation

Whole blood (10 ml.) or its equivalent of plasma is diluted to 200 ml. with water, conc. HCl (11 N, 30 ml.) is added and the mixture is boiled under a reflux condenser for 1 hr. After cooling under running water, the hydrolysed blood is treated exactly as the hydrolysed urine in method A up to the chromatography stage. During the extractions with ether, care is taken not to shake too vigorously. The emulsion which forms during the first ether extraction is centrifuged at 2000 rev./min for 20 min when it separates into three layers. The ether and aqueous layers are decanted separately and the layer of dark brown material is discarded (separation into layers is facilitated if the hydrolysed blood has been kept at 4° C overnight before extracting with ether). The emulsions which form during the second and third ether extractions usually separate when given a vigorous shake after the aqueous layer has been run off. Emulsions also form during the extraction of the oestrone and oestradiol fraction from the mixture of light petroleum and benzene with 1·6 % NaOH. These emulsions separate, however, on standing for 10–20 min. The layer of interfacial solid which forms at this stage is discarded.

After methylation and the addition of hydrogen peroxide and NaOH, the methylated oestriol fraction is extracted with 25 ml. benzene, and the methylated oestrone/oestradiol fraction is extracted with 25 ml. light petroleum, the solvents being used first to rinse the corresponding methylation flasks. The benzene and light petroleum extracts are washed twice with two 5 ml. vol. water and the water is drained off as completely as possible.

Chromatography

Oestriol methyl ether fraction. A column is prepared by pouring 0·7 g of standardized deactivated alumina into a chromatography tube partly filled with benzene. The alumina is allowed to settle and a protective 5 mm layer of sand is added. The benzene extract containing the oestriol methyl ether is applied to the column, taking care not to transfer any droplets of water with it. When all the benzene has percolated through, the column is eluted with 3 ml. 1·4 % ethanol in benzene, the eluate being discarded. The column is then eluted with 7 ml. 2·5 % ethanol in benzene; the eluate contains the oestriol methyl ether and is collected into a tube for evaporation and development of the Kober colour. Fractions from foetal blood contain relatively large amounts of oestriol and suitable aliquots are required for colorimetry; or preferably the Kober reaction is performed with 3 ml. of reagent.

Oestrone/oestradiol methyl ether fraction. This fraction is similarly applied to another column of alumina (0·7 g) prepared in light petroleum. The column is eluted first with 4 ml. 25 % benzene in light petroleum which is discarded. It is then eluted with 10 ml. 40 % benzene in light petroleum; the first 7 ml. of this eluate contains the oestrone methyl ether and is collected, the last 3 ml. is discarded. The column is then

eluted with 5 ml. benzene; the eluate contains the oestradiol methyl ether and is collected.

Evaporation of solvents. Quinol (0·5 mg) in ethanolic solution (0·05 ml. of 1 %w/v) and a small piece of clean porous tile or alundum are added to each eluate and to appropriate blank tubes. The solutions are evaporated in an atmosphere of nitrogen, the rate being controlled by applying a partial vacuum from a water pump. When a few ml. of solvent remain, the tubes are raised in the water bath so that the remainder distils slowly and the residue collects in the narrow end of the tube. Finally, all traces of solvent are removed. This is ensured by allowing the tubes to stand for a few seconds in the boiling water bath under full vacuum from the water pump. The tubes are then allowed to cool under a stream of nitrogen. Any possible contamination with dust particles, etc., at this stage should be excluded by using scrupulously clean glassware and keeping the tubes covered.

Colour development and colorimetry. After evaporation, 0·3 ml. of the appropriate Kober reagent is added to each tube (2 %, w/v, quinol in 76 %, v/v, H_2SO_4 to the oestriol tubes, 2 % quinol in 66 % H_2SO_4 to the oestrone tubes and 1·5 % quinol in 60 % H_2SO_4 to the oestradiol tubes). The reagent is made to flow over the lower walls of the tubes in order to dissolve all the visible residue. The tubes are then heated for 20 min in a boiling water bath, the contents being carefully mixed once during the first 5 min of heating. They are then cooled for approx. 10 min in a bath of cold water, and 0·1 ml. water is added to the oestriol tubes, 0·05 ml. to the oestrone tubes and 0·02 ml. to the oestradiol tubes. The contents are carefully but thoroughly mixed, and the tubes are heated for a further 10 min in the boiling water bath. They are then cooled again in cold water for approx. 10 min. Optical densities are measured against similarly treated reagent blanks in the spectrophotometer at the following wavelengths: oestriol and oestrone fractions, 480, 516 and 552 mμ; oestradiol fraction, 480, 518 and 556 mμ.

Optical density readings (D) are corrected by applying the following formulae which are derived from that of Allen (1950).

Oestriol and oestrone corrected readings $= 2D_{516} - (D_{480} + D_{552})$.
Oestradiol corrected reading $= 2D_{518} - (D_{480} + D_{556})$.

The amount of oestrogen methyl ether present in each tube is obtained by applying the corrected reading to the appropriate standard calibration graph, and is converted to the corresponding amount of unmethylated oestrogen by multiplying by the factor 0·95 (the ratio of the molecular weights). Standard graphs are prepared at approx. 6-weekly intervals using pure oestrogen methyl ethers (0·1–0·8 μg); quinol (0·5 mg) is added to each tube together with the solvent used for eluting that particular oestrogen from the alumina columns, i.e. 2·5 % ethanol in benzene (7 ml.) to the oestriol tubes, 40 % benzene in light petroleum (7 ml.) to the oestrone tubes and benzene (5 ml.) to the oestradiol tubes; only the quinol in ethanolic solution (0·05 ml.) is added to the blank tubes; the solvents are evaporated and the Kober colours are developed exactly as described above. The corrected readings obtained over a 2-year period are summarized in Table 1. Also shown are the values obtained when solvents are not added. The solvent contributes to the Kober-chromogenic impurities (see Table 4), but does not affect significantly the corrected optical densities of the standards.

RESULTS

(1) *Accuracy of method*

Recovery experiments were performed to test the accuracy of the extraction, purification, and colorimetric stages of the method and to determine whether losses occur during acid hydrolysis. Samples of blood were obtained from men and non-pregnant women, and from women who were 30–40 weeks pregnant. Foetal blood

Table 1. *Relationship between corrected spectrophotometer readings and amounts of oestrogen methyl ethers (expressed in terms of weight of the free oestrogens)*

Oestrogen (μg)	Corrected readings (mean ± S.D.)		
	Oestriol	Oestrone	Oestradiol
0·1	0·050 ± 0·009 (14)	0·057 ± 0·009 (14)	0·046 ± 0·011 (13)
0·2	0·099 ± 0·013 (14)	0·127 ± 0·021 (14)	0·118 ± 0·010 (13)
0·4	0·185 ± 0·011 (16)	0·265 ± 0·024 (15)	0·244 ± 0·019 (15)
*0·4	0·167 ± 0·018 (5)	0·256 ± 0·020 (8)	0·248 ± 0·012 (16)
0·8	0·350 ± 0·023 (15)	0·503 ± 0·046 (12)	0·487 ± 0·026 (12)

All values shown were obtained with added solvent except those marked * where the standards were evaporated from 0·4 ml. ethanol (see Table 4). No. of observations in parentheses.

Table 2. *Recovery of oestrogens added to blood*

(All figures corrected for endogenous values.)

Source of blood	Stage when oestrogens were added	Oestriol			Oestrone			Oestradiol		
		μg added/ 10 ml.	No. of obs.	% recovery (mean ± S.D.)	μg added/ 10 ml.	No. of obs.	% recovery (mean ± S.D.)	μg added/ 10 ml.	No. of obs.	% recovery (mean ± S.D.)
Non-pregnant individuals	After hydrolysis	0·05	7	54 ± 20	0·05	10	78 ± 13	0·05	10	69 ± 15
Pregnant women	After hydrolysis	1·0	13	63 ± 8	0·4	12	66 ± 13	0·2	12	67 ± 13
	Before hydrolysis	1·0	6	56 ± 4	0·4	7	56 ± 5	0·2	8	47 ± 5
Umbilical cord	After hydrolysis	16·0	13	64 ± 7	0·4	17	68 ± 9	0·2	23	72 ± 11
	Before hydrolysis	16·0	11	59 ± 7	0·4	14	65 ± 12	0·2	16	64 ± 14

was obtained from the umbilical cord at delivery. Each sample was divided into two portions and the three oestrogens were added before or after hydrolysis to one portion, in amounts corresponding to those found during pregnancy. The two portions were assayed and the recovery of the added oestrogen was calculated after subtracting the endogenous values. The results are summarized in Table 2. When oestrogens were added after hydrolysis, approx. 64 % of the oestriol, 67 % of the oestrone and 69 % of the oestradiol was recovered; when added before hydrolysis, approx. 59 % of each of the three oestrogens was recovered, indicating that small losses occur during the boiling with acid. These figures are lower than those for urine where, for example, 80–85 % of the oestrogen added after hydrolysis is recovered. Attempts to improve these figures for blood were unsuccessful. Plasma gave the same results. The losses appear to be due to partial adsorption on to the hydrolysed

protein, to interference in partition coefficients by the blood lipids and to mechanical difficulties caused by the emulsions and interfacial solids which form during the procedure.

(2) *Precision of method*

An estimate of the precision of a method is given by the standard deviation (S.D.) of results of replicate determinations from their means. This was calculated by the method of Snedecor (1952) from a series of duplicate analyses as follows:

$$\text{estimate of S.D. } (`s') = \sqrt{\frac{\Sigma d^2}{2N}},$$

where d is the difference between the two results in a duplicate determination, and N is the number of duplicate determinations performed.

Table 3 summarizes the values of 's' calculated in this manner. The results are given for various levels of oestrogen concentration and are expressed as μg/100 ml. blood. Values for 's' depend on oestrogen concentration. A number of useful factors

Table 3

A. *Precision expressed as estimates of* S.D. ('s') *of results from their means* (*from duplicate determinations*)

	Source of blood	No. of duplicate determinations	Oestrogen concn. (μg/100 ml. blood)	's' (μg/100 ml. blood)
Oestriol	Pregnant women	37	0–1·0	0·13
	Pregnant women	91	1·1–10·0	0·22
	Umbilical cord	16	50–90	5·8
Oestrone	Pregnant women	71	0–1·0	0·08
	Pregnant women	66	1·1–7·0	0·19
	Umbilical cord	21	0–1·5	0·21
Oestradiol	Pregnant women	92	0–0·5	0·06
	Pregnant women	44	0·6–1·3	0·08
	Umbilical cord	21	0–1·6	0·17

B. *Sensitivity and fiducial range calculated for a duplicate determination from* 's' ($P = 0{\cdot}05$)

(Values in μg/100 ml. blood.)

Pregnancy blood	Oestriol	Oestrone	Oestradiol
Smallest amount distinguishable from zero	0·18	0·11	0·08
Smallest amount measured with an error of $< \pm 25\,\%$	0·72	0·45	0·34
Fiducial range*			
Concn. 1·0 μg/100 ml. or less	M $\pm$ 0·18	M $\pm$ 0·11	M $\pm$ 0·08
Concn. > 1·0 μg/100 ml.	M $\pm$ 0·31	M $\pm$ 0·27	M $\pm$ 0·11
Umbilical cord blood			
Smallest amount distinguishable from zero	8·0	0·3	0·2
Smallest amount measured with an error of $< \pm 25\,\%$	32	1·2	0·8
Fiducial range	M $\pm$ 8·0	M $\pm$ 0·3	M $\pm$ 0·2

* Cf. text p. 15.

can be calculated from this estimate. For example, the smallest amount of oestrogen which is distinguishable from zero can be calculated as $ts/\sqrt{N}$; the least amount of oestrogen which is determined with an error of say $\pm 25\,\%$ can be calculated as $100ts/25\sqrt{N}$; and the fiducial range of a result can be calculated as $M \pm ts/\sqrt{N}$, where M is the mean of N determinations. When analyses are performed in duplicate and $P = 0{\cdot}05$ ($t = 1{\cdot}96$) these calculations give the values shown in Table 3. It should, however, be remembered that these calculations give an indication of the basic handling errors of the method, but do not take into account the errors due to incomplete extraction from blood or any lack of specificity of the method.

(3) *Specificity of method*

The specificity of the method depends on the Kober colour reaction and on the extraction and purification procedures employed.

The formation, in the Kober reaction used here, of an intense pink colour with an absorption maximum at 516–518 mμ, with the characteristic wavelength–absorption curve illustrated in Fig. 1, and a high extinction coefficient ($E\,{}^{1\%}_{1\,cm}$) of approx. $1{\cdot}5 \times 10^3$ at 516–518 mμ, is a specific property of oestriol, oestrone and oestradiol, and of some of the other closely related natural oestrogens. This colour should not be confused with a slight deviation from linearity given by some impurities in the region of 516 mμ. No other phenolic compounds are known to give this colour reaction.

The extraction and purification procedures contribute to the specificity of the method by removing non-phenolic compounds and most of the other impurities, and by separating the oestrogens from one another. During chromatography, each oestrogen fraction from blood is normally collected in one portion of eluate; however, when it is fractionally eluted, the substance being measured as oestrogen shows exactly the same elution pattern as the corresponding pure oestrogen methyl ether (Table 4). The procedure does not completely separate 16-*epi*oestriol from oestriol so that a trace of this oestrogen contaminates the oestriol fraction (Brown *et al.* 1957*b*); nor does it separate equilin and equilenin from oestrone, or oestradiol-17α from oestradiol-17β (Brown, 1955), but these three oestrogens are not known to occur in the pregnant woman. The α-ketolic oestrogens such as 16α-hydroxyoestrone are destroyed during the method and do not interfere. Some of the 2-methoxyoestrone appears after methylation in the oestradiol methyl ether fraction; however, this oestrogen gives an atypical Kober colour which is eliminated by the spectrophotometric correction.

The spectral characteristics of the Kober colours produced by the pure oestrogen methyl ethers and by blood extracts which have been prepared by the method are illustrated in Fig. 1. The three fractions from pregnancy blood all show a peak of absorption at 516–518 mμ which indicates that they contain oestrogen. The fraction from non-pregnancy blood shows no peak in this region, its wavelength–absorption curve being practically linear between 480 and 560 mμ (corrected reading $-0{\cdot}004$). By comparison with the pure oestrogen methyl ethers, the fractions from blood also contain a yellow component which absorbs most strongly at the shorter wavelengths. This component interferes in the direct optical density measurements of any oestrogen pink colour which may be present. If the fraction from non-pregnancy blood shown

Table 4. *Elution pattern of oestrogen methyl ethers and blood extracts from alumina* columns*

Eluate	Benzene-light petroleum						Benzene		
	25/75	40/60							
ml. of eluate	4	2	4	6	8	10	2	4	6
Oestrone methyl ether	—	24	43	31	2	—	—	—	—
Oestradiol-17β methyl ether	—	—	—	—	—	—	24	62	14
Blood extract	—	29	42	21	8	—	38	54	8

Eluate	Ethanol-benzene				
	1·4/98·6	2·5/97·5			
ml. of eluate	3	2	4	6	8
Oestriol methyl ether	—	6	64	28	2
Blood extract	—	2	91	5	2

Results are expressed as percentage of oestrogen present in each fraction, and in the case of blood extracts refer only to Kober chromogens estimated as oestrogens after applying the colour correction.
* Activity as specified by Brown (1955).

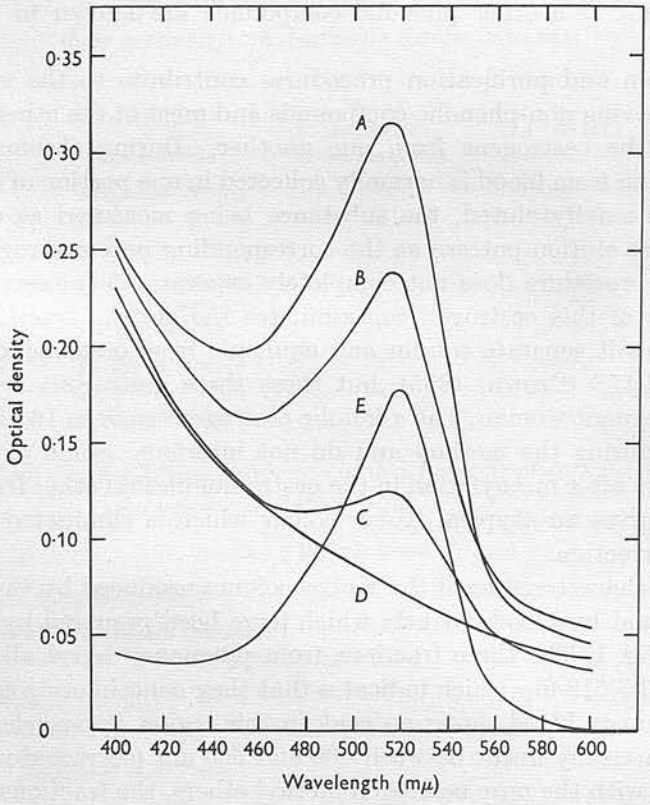

Fig. 1. Wavelength–absorption curves of colours produced in the micro-Kober reaction. *A*, oestriol; *B*, oestrone; and *C*, oestradiol fractions from pregnancy blood; *D*, oestradiol fraction from non-pregnancy blood; *E*, pure oestradiol methyl ether (0·4 μg). Corrected readings were *A*, 0·296; *B*, 0·198; *C*, 0·080; *D*, −0·004; *E*, 0·246.

in the figure contains no detectable oestrogen, which is likely, then its wavelength–absorption curve represents that of the yellow impurity. Since this curve is linear between 480 and 560 mμ, the interference in the optical density measurements is completely eliminated in this particular example by the Allen spectrophotometric correction. The specificity of the method depends on whether the impurities always conform to these spectral characteristics. Consequently, the Kober colours produced by a large number of extracts from blood and plasma and by residues from evaporated solvents were analysed. The basic assumption was made that the wavelength–absorption curves of the impurities *were linear* between 480 and 560 mμ, and from this the amounts of impurities present were calculated by the Allen formula as follows:

$$D \text{ impurities} = \text{observed } D_{\max.} - \text{corrected reading} \times 0{\cdot}75,$$

where *D impurities* is the optical density at 516–518 mμ contributed by the impurities, *observed* $D_{\max.}$ is the observed optical density at 516–518 mμ, the *corrected reading* is calculated from the optical density measurements at 480, 516–518 and 552–556 mμ,

Table 5. *Kober-chromogenic impurities in oestrogen fractions*

	Calculated optical densities of impurities at 516–518 mμ (mean ± S.D./tube)		
	Oestriol	Oestrone	Oestradiol
A. 'Pure' solutions			
Pure oestrogen methyl ethers (0·4 μg in 0·4 ml. ethanol)	0·005 ± 0·028 (5)	−0·006 ± 0·030 (8)	0·012 ± 0·022 (16)
Solvents* together with pure oestrogen methyl ethers (μg oestrogen)			
0·1	0·055 ± 0·035 (14)	0·090 ± 0·036 (14)	0·061 ± 0·023 (13)
0·2	0·082 ± 0·035 (14)	0·092 ± 0·034 (14)	0·078 ± 0·034 (13)
0·4	0·075 ± 0·030 (16)	0·101 ± 0·036 (15)	0·081 ± 0·031 (15)
0·8	0·109 ± 0·025 (15)	0·112 ± 0·048 (12)	0·098 ± 0·067 (12)
Combined range 0·1–0·8 μg	0·081 ± 0·036 (59)	0·098 ± 0·038 (55)	0·079 ± 0·042 (53)
B. Extracts from blood (10 ml.)			
Range of oestrogen content (μg/10 ml.)			
0–0·2	—	—	0·088 ± 0·037 (154)
0–0·7	—	0·100 ± 0·039 (147)	—
0–1·0	0·070 ± 0·032 (152)	—	—

No. of observations in parentheses.

* Solvent added to the oestriol tubes was 2·5 % ethanol in benzene (7 ml.); to the oestrone tubes, 40 % benzene in light petroleum (7 ml.); to the oestradiol tubes, benzene (5 ml.); none was added to the blank tubes. All tubes contained quinol (0·5 mg) added in ethanolic solution (0·05 ml.).

as described in the method section, and the factor *0·75* is the ratio observed $D_{\max.}$/corrected reading for the pure oestrogen methyl ethers when the Kober reaction is performed with 3 ml. of reagent (Brown, 1955).

The results of these calculations are summarized in Table 5, those for the 'pure' solutions being derived from the same readings as the standards given in Table 1.

On average, practically no impurities were present when the pure oestrogen methyl

ethers were evaporated directly from 0·4 ml. of ethanol, as might be expected; however, considerable amounts of impurities were found when the pure oestrogen methyl ethers were evaporated with the same volumes of solvents as those used for eluting the fractions from the alumina columns, and these amounts of impurities were no less than those found in the extracts from blood. The Kober chromogenic impurities present in blood extracts were therefore derived entirely from the solvents used in the assay procedure and not from the blood; their contribution to the optical densities at 516–518 mμ is, on average, equivalent to that produced by 0·21 μg oestriol, 0·21 μg oestrone and 0·19 μg oestradiol per tube. This source of contamination had not been recognized in the related urinary methods, and had become apparent here only because of the exaggerating effect of the small volumes of reagents used in the Kober reaction. When the oestrogen methyl ethers were added directly to the solvents, the amounts of the impurities tended to increase with increasing oestrogen concentration. However, only in the case of oestriol was this increase significant; it was almost certainly due to a small error in the factor 0·75 used in the calculations and does not affect the general conclusions. Considerable variations in the amounts of impurities occurred within each group and within replicates from the same source. However, the variation within groups was approximately the same for all the groups (S.D.'s between 0·03 and 0·04), a finding which indicates that the variation had a common cause in all the groups.

Attempts to remove the impurities from the solvents were unsuccessful, apart from a possible improvement in the ethanol by standing over *m*-phenylenediamine. The amounts of impurities were diminished by decreasing the size of the alumina columns and consequently decreasing the volumes of eluants required, and also by decreasing the amounts of quinol added. In fact, traces of quinol deposited on the walls of the tubes during evaporation, and exposed to variable degrees of oxidation, could well be the major source of the extraneous colours. This would explain the variations in the amounts of impurities found, which also occurs in the blank tubes, as well as the fact that large volumes of solvents produce larger amounts of impurities because they extend the area covered with quinol. Dirty glassware and particles of dust, rubber or cork are other possible sources of contamination which must be rigidly excluded after chromatography.

Since the impurities are derived from the procedure and not from the blood, the spectral characteristics of the colours they produce can be more readily defined. A simple method for eliminating their effect would be to add solvents to the blank tubes. However, the amounts of impurities vary so much that this expedient often leads to negative optical density readings. As shown in Table 1, the corrected optical densities of the oestrogen methyl ether standards are the same whether the solvent with its impurities is added or not; this could happen only if the wavelength–absorption curves of the impurities are in fact linear between 480 and 560 mμ. Allen's spectrophotometric correction is therefore a completely valid procedure for eliminating this interference. Furthermore, the addition of solvents to the standards used for preparing calibration graphs provides a further safeguard against errors arising from this source. Under these conditions the Kober colour reaction confers a high degree of specificity on the results obtained.

(4) *Application of additional purification procedures*

A twofold purification of the oestrogen fractions from urine is achieved by adding a saponification step to the method, and a further fivefold purification of the oestrone fraction is achieved through the complex formed with Girard's Reagent T (Bauld, 1956; Brown *et al.* 1957*a*; Brown & Blair, 1960). These procedures, which increase the reliability of the method for urine, have been incorporated in the method for blood, with the results shown in Table 6. Cord blood was used for this comparison because of its availability, and oestriol was not estimated because its concentration is sufficiently high in this fluid for there to be little doubt about the validity of the results obtained.

Table 6. *Effect of additional purification procedures on oestrone and oestradiol fractions from cord blood**

Purification procedure	Whether applied	No. of comparisons	Change† in oestrogen values (μg/100 ml.) Oestrone	Oestradiol	Impurities $D_{516-518}$/10 ml. blood Oestrone fraction	Oestradiol fraction
Saponification	Yes	7	$-0{\cdot}02 \pm 0{\cdot}09$	$-0{\cdot}04 \pm 0{\cdot}07$	$0{\cdot}051 \pm 0{\cdot}023$	$0{\cdot}051 \pm 0{\cdot}027$
	No	7			$0{\cdot}061 \pm 0{\cdot}022$	$0{\cdot}077 \pm 0{\cdot}022$
Girard separation	Yes	8	$-0{\cdot}01 \pm 0{\cdot}08$	—	$0{\cdot}080 \pm 0{\cdot}051$	—
	No	8			$0{\cdot}082 \pm 0{\cdot}025$	—

* Containing 0·6–2·1 μg oestrone and 0–0·6 μg oestradiol/100 ml.
† Results with additional purification minus results without additional purification.

Compared with the basic method, the additional purification steps did not alter the oestrogen values or the amounts of impurities found in the final fractions. This agrees with other conclusions regarding the method; if oestrone and oestradiol were not being measured specifically, the results might have been altered through removal of contaminating material, and if the impurities were derived in part from the blood, then these, too, should have been altered by the additional purification procedures.

(5) *Comparison between the oestrogen content of whole blood and plasma*

Seven 40 ml. pools of blood were collected from pregnant women. Each pool was divided into two 20 ml. portions and the oestrogen content of each portion was determined in duplicate (1) directly using the whole blood, and (2) after separating the plasma and removing the red cells, as described in the Methods section. According to the criteria listed in Table 3, no result for the plasma (plus saline washings of the red cells) differed significantly from that for the equivalent volume of whole blood. The mean differences, blood minus plasma, in μg/100 ml. blood, with their S.D.'s were: $-0{\cdot}12 \pm 0{\cdot}12$ for oestriol, $-0{\cdot}11 \pm 0{\cdot}13$ for oestrone and $-0{\cdot}08 \pm 0{\cdot}04$ for oestradiol, the ranges of concentration measured being, respectively, 1–6, 0·6–4·3 and 0·2–1·0 μg/100 ml. blood.

DISCUSSION

Werthessen, Baker & Field (1950) reported losses of oestrone during incubation with whole blood amounting to 40–70 % of that added. This great loss has not since been confirmed (Veldhuis, 1953), but Gray & Bischoff (1955) have shown that there

is a 4–5 % conversion of oestrone to oestradiol during incubation with whole blood. This conversion of added oestrone has been confirmed by Wall & Migeon (1959), who have shown that the change is insignificant when the period of incubation is 30 min or less, and that it does not occur in plasma alone. Many workers estimating blood oestrogens have taken precautions against these changes by separating the plasma soon after collecting the blood and storing it in the frozen state until assay (Aitken, Preedy, Eton & Short, 1958). Consequently, most results have been reported for plasma rather than for whole blood.

In vitro and *in vivo* studies with ^{14}C-labelled oestrone and oestradiol show that up to 25 % of the total blood oestrogens may be associated with the red cells (Sandberg & Slaunwhite, 1957; Wall & Migeon, 1959; Migeon, Wall & Bertrand, 1959). These would not be included in assays using plasma alone. However, these oestrogens, which appear to be adsorbed on the red cells, can be removed by successive washing of the red cells with plasma or saline, the first washing being the most effective (Wall & Migeon, 1959). In the present work all assays on plasma included the saline washings of the red cells, and under the conditions used no differences could be detected between the oestrogen values for plasma and those for the equivalent volume of whole blood, although slightly lower values for plasma might have been expected since the washing procedure should not have been complete. Other experiments were performed to determine whether any changes in the endogenous oestrogen content of blood could be detected on storage. Specimens of pregnancy blood were divided into two portions, one was processed immediately and the other after being stored at 4° C for several days. The values obtained were identical, indicating that endogenous oestriol, oestrone and oestradiol are stable in whole blood when kept at 4° C. The extra labour involved in separating plasma from the red cells is therefore unnecessary. Furthermore, blood tends to give less troublesome emulsions and interfacial solids during the assay procedure. Therefore, on the basis of convenience, whole blood is preferred to plasma for the estimation of oestrogens.

The oestrogens are present in blood in a variety of forms which have been defined as 'free', 'conjugated' and 'protein-bound'. The accuracy of a method depends on the efficiency with which these forms are extracted from blood and converted to the free oestrogens for assay. A considerable amount of work was performed to investigate this point. However, when each oestrogen had been divided into its various fractions, the amounts present in each fraction were usually too small to be measured with any accuracy and small changes in yield with differing fractionation procedures could not be detected. All that could be done was to determine which procedure gave the maximum yield of each oestrogen from blood. Of all the methods investigated, dilution with water followed by boiling with 15 vol. % concentrated hydrochloric acid gave the highest yields, although losses occurred during the process. Similar conditions have been selected by Varangot *et al.* (1955), Preedy & Aitken (1957), and Oertel, West & Eik-Nes (1959). A method which distinguishes between the oestrogen content of the plasma and red cells, and between 'free', 'protein-bound' and 'conjugated' oestrogens would have distinct advantages over the present methods which determine total oestrogens only. However, a much more sensitive method of detection and measurement than the micro-Kober colour reaction used here is necessary for the purpose. The fluorimetric method of Ittrich (1958), which is a

modification of the Kober reaction, might provide this sensitivity together with the necessary specificity.

The present method recovers approx. 65 % of oestriol, oestrone and oestradiol added to blood or plasma after acid hydrolysis. The corresponding figure for urine is approx. 82 %. Since the extraction and purification procedures are identical, the lower recoveries must be caused by the constituents of the blood; for example, losses could occur through partial adsorption on to the hydrolysed protein, through interference in partition coefficients by the blood lipids and through mechanical difficulties caused by emulsions and interfacial solids which form during the procedure. Similar difficulties have not been reported by other workers. Veldhuis (1953) obtained recoveries of 80–90 % for his method; Varangot *et al.* (1957) obtained recoveries of 68–75 %; and Preedy & Aitken (1957) reported that the recovery of oestrogens from plasma by their method was the same as from urine (approx. 80 %). There is at present no satisfactory explanation for this discrepancy.

The oestriol, oestrone and oestradiol contents of blood and plasma obtained from pregnant and non-pregnant individuals and of foetal cord plasma, have been reported by several workers. Reliable figures for a pool of late pregnancy plasma have also been published by Oertel *et al.* (1959), the oestrogens having been isolated and identified by an exhaustive process which included countercurrent distribution, paper chromatography, the Kober reaction and infrared spectrophotometry. These results are summarized in Table 7 together with those obtained by the present method.

There are good reasons for accepting the reliability of the results obtained by Preedy and his co-workers, and by Oertel *et al.* (1959) for pregnancy and foetal plasma. When allowance is made for the fact that plasma occupies approximately half the blood volume and contains most of the oestrogen, there is excellent agreement between their results for plasma and those obtained by the present method for blood. In spite of the many assumptions underlying the corrections for impurities used by Veldhuis (1953), his values also agree very well. The figures reported by Varangot *et al.* (1956) are obviously much too high, and illustrate the errors involved in the direct fluorimetric measurement of oestrogens in relatively crude blood extracts without taking additional precautions to ensure specificity. The results for cord blood obtained by the present method agree closely with those reported by Diczfalusy & Magnusson (1958). Preedy & Aitken (1957) found small but detectable amounts of oestriol and oestrone in some plasmas from non-pregnant individuals. Similar values for oestrone were found by the present method in some bloods from this source. However, the amounts were not greatly different from zero, and the error of the estimation at these levels is large. Whether these values do in fact represent oestrone has yet to be determined.

The method has been in use in this laboratory for 2 years and has proved satisfactory for the study of oestrogen levels in blood during normal and abnormal pregnancies from the 12th week to term and in foetal blood at birth. A preliminary communication describing the results of this application has been published (Kellar, Matthew, Mackay, Brown & Roy, 1959).

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Table 7. *Comparison of results reported for various methods*

	No. of observations	Oestrogen concn. (μg/100 ml.; ranges and means)		
		Oestriol	Oestrone	Oestradiol
Pregnant women				
Blood, 8–8½ months (Varangot *et al.* 1956)	8	24–280 (156)	11–155 (59)	
Plasma, 7–8 months (Veldhuis, 1953)	3	1·0–1·6 (1·4)	2·1–3·3 (2·7)	
Plasma, 3rd trimester (Oertel *et al.* 1959)	1	2·9	5·2	3·3
Plasma, 38 weeks to labour (Preedy & Aitken (1957); Aitken *et al.* (1958))	11	4·3–19·8 (13·3)	2·7–17·8 (9·0)	0·6–3·7 (2·0)
Blood, 36 weeks to term (Roy & Brown, present study)	19	2·3–19·6 (7·8)	0·5–9·0 (5·0)	0·2–1·7 (1·0)
Foetus at birth (cord blood)				
Plasma (Aitken *et al.* 1958)	5	59–229 (129)	1·0–6·1 (3·6)	< 0·2–0·8 (0·5)
Blood (Diczfalusy & Magnusson, 1958)	9	39–68 (58)	0–2·3 (1·3)	0·1–1·3 (0·6)
Blood (Roy & Brown, present study)	22	30–70 (49)	0·7–2·3 (1·5)	0–1·5 (0·6)
Non-pregnant subjects				
Blood, menstrual cycle (Varangot *et al.* 1956)	39	0–32 (4)	12–190 (73)	
Blood, men and postmenopausal women (Varangot *et al.* 1956)	8	0	70–160 (110)	
Plasma pooled (Veldhuis, 1953)	1	0·39	0·31	
Plasma, women luteal phase (Preedy & Aitken, 1957)	6	< 0·15, 0·21, 0·30	< 0·06, 0·13, 0·14, 0·22	< 0·07
Plasma, men (Preedy & Aitken, 1957)	5	< 0·15	< 0·06, 0·11	< 0·07
Blood, pooled and menstrual cycle (Roy & Brown, present study)	6	< 0·18	< 0·11, 0·13, 0·18, 0·30	< 0·08

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