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OESTROGEN AND OESTROGEN CONJUGATE CONCENTRATIONS IN LATE PREGNANCY PLASMA

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SUMMARY

Oestrogen and oestrogen conjugate concentrations have been measured in plasma samples withdrawn at intervals during late pregnancy. Unconjugated oestrogens, separated on Sephadex LH-20, were measured by competitive binding to rabbit uterine cytosol. Procedural losses were offset by the addition of internal standards. A wide variety of synthetic oestrogen conjugates showed little or no capacity to displace radioactive oestradiol from the binding protein and for this reason plasma oestrogen conjugates were determined indirectly by competitive binding after hot acid hydrolysis. Oestriol conjugates were predominant in the conjugated oestrogen fraction; oestradiol and oestriol were major components of the unconjugated oestrogen fraction. Plasma oestrogen concentrations are more readily determined than urinary oestrogen values and if the analysis is carried out on serial samples the result reflects changes more rapidly and faithfully than the longer urine method.

THE ADVENT of competitive binding methods [1, 2] has now provided a simple assay of adequate sensitivity and specificity for the measurement of oestrone, oestradiol and oestriol in pregnancy plasma. Unfortunately, oestrogen conjugates do not bind to this naturally-occurring oestrogen receptor site with high affinity and these combined forms at present must be determined after hydrolysis. The plasma concentration of oestrone, oestradiol and oestriol as free compounds in late pregnancy has been measured; the corresponding conjugates have been hydrolysed and the released oestrogens have been separated and determined. Duplicate assays showed good agreement; procedural losses have been off-set by the use of radioactive internal standards.

MATERIALS AND METHODS

Oestrogens of high specific radioactivity were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. [2,4,6,7-^{3}H]-oestrone (100 Ci/mmol), [2,4,6,7-^{3}H]-oestradiol (100 Ci/mmol) and [2,4-^{3}H]-oestriol (10 Ci/mmol) were checked for purity at intervals by t.l.c. on silica gel in two systems (cyclohexane-ethyl acetate 65:35 and chloroform-acetone 9:1 (v/v)) and, when necessary, were purified by gel filtration on Sephadex LH-20. Stock solutions were prepared in benzene (20 ng/ml stored at 6–10°C) and were diluted as standard solutions as required. Gel filtration of oestrogens was carried out on Sephadex LH-20 columns by the method of Mikhail *et al.* [3].

Non-radioactive oestrogens, obtained from Sigma (London) Chemical Co., London, S.W.6, U.K. were chromatographically pure and were stored in stock solutions in ethanol (0·4 mg/ml). Standard solutions containing 10–200 pg/0·1 ml prepared by diluting stock solutions with buffer were stable for at least 2 weeks.

Buffer solution contained 10 mM-tris-HCl pH 8·0, 1 mM-EDTA and 250 mM sucrose.

Dextran-charcoal suspension used to remove unbound oestrogens contained 0·25% Norit A in 0·0025% dextran (Mol. wt. 60,000–100,000) in buffer.

Preparation of uterine supernatant. Virgin New Zealand white rabbits (2–2·5 kg) were used as a source of binding protein and as the oestrogen binding capacity of supernatant preparations varied considerably preliminary experiments were carried out to find the optimum dilution for the competitive binding assay [4]. The preparations after appropriate dilution with buffer were stored at −10°C in the presence of 0·1 M-mercaptoethanol.

Scintillation counting. Samples in aqueous phase (0·05–0·5 ml) were counted in a Packard Model 3375 spectrometer in 10 ml of toluene scintillation liquid (0·01% POPOP, 0·4% PPO); the contents of the vial were mixed on a whirlimixer agitator (Fisons Scientific Apparatus Co. Ltd., Loughborough, Leics., U.K.) and were allowed to stand in the dark for 2h to allow separation of the phases before counting (efficiency ~ 35%). Non-aqueous samples were counted after mixing.

Determination of unconjugated oestrogens in plasma

The method of oestrogen binding assay used was, in principle, that of Korenman [2]. Separation of unconjugated oestrogens and of oestrogens liberated by conjugate hydrolysis was achieved by reverse phase gel filtration on Sephadex LH-20 [3]. By plotting the reciprocal of the radioactivity bound against increasing amounts of non-radioactive oestrogen, linear calibration graphs were obtained for oestradiol (0-200 pg), oestrone (0–300 pg), oestriol (0–500 pg) by displacement of tritium-labelled oestradiol (9,000 d.p.m.) from the uterine supernatant preparation (Fig. 1).

The plasma sample (1 ml) was transferred to a centrifuge tube (15 ml) containing the following radioisotope-labelled internal standards: oestrone (~ 8 pg,

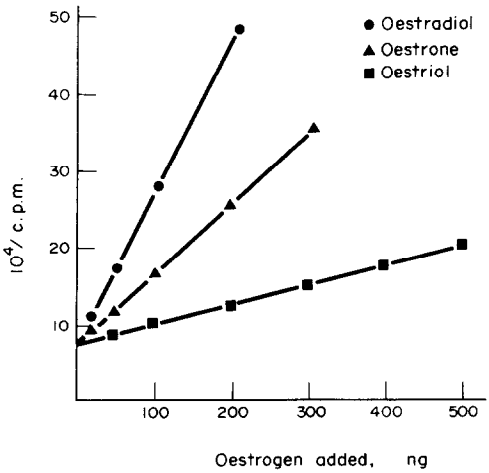


Fig. 1. Calibration graphs for oestradiol (●——●), oestrone (▲——▲) and oestriol (■——■) by competitive binding assay to rabbit uterine cytosol in the presence of [2,4,6,7-^{3}H]oestradiol (100 Ci/mmol). The ordinate represents the reciprocal of bound counts and the abscissa increasing amounts of non-radioactive oestrogen.

6,000 d.p.m.), oestradiol (~ 12 pg, 9,000 d.p.m.) and oestriol (~ 50 pg, 3,000 d.p.m.) and the tube contents were mixed on an agitator and stood for 15 min. After extraction with ether (1 × 10 ml, 1 × 5 ml) and chromatographic separation of the dried extract on Sephadex LH-20, peak fractions only were combined and taken to dryness. A portion of each was set aside for counting in order to determine the percentage recovery of the intrinsic oestrogen. This selection of peak fractions had the advantage of reducing the likelihood of contamination of the oestrogens with each other yet the concentration of unconjugated oestrogens in late pregnancy plasma is such that these peak fractions, even when half the material was used for measurement of the internal standard, contain more than sufficient oestrogen for the competitive binding assay.

For oestrone the combined peak fractions were taken to dryness and the residue was dissolved in buffer (1 ml). Of this solution 0·5 ml was used to determine the recovery of unconjugated oestrone and the remainder was further diluted by the addition of an equal volume of buffer. The 100 μl samples used for competitive assay in triplicate represented one twentieth of the recovered oestrone. The recovered oestradiol was diluted by a factor of 1 in 150 to bring it within the calibration range. The oestradiol extract was dissolved in buffer (1 ml) and half of this solution was used for recovery measurement. A portion of the remainder (0·1 ml) was diluted with buffer to 1·5 ml and the assay was carried out in triplicate on 100 μl portions. For unconjugated oestriol determination a dilution of 1 in 20 was necessary and this dried residue was treated in the same manner as that of oestrone.

Determination of oestrogen conjugates in plasma

The failure of oestrogen conjugates to compete with radioactive oestradiol for oestrogen receptor sites. A wide range of synthetic oestrogen conjugates has been tested for the ability to compete with radioactive oestradiol for the high-affinity oestrogen-binding sites of rabbit uterine supernatant preparations. These tests were carried out by the calibration procedure and included the following oestrogen monoglucuronosides; oestrone-3-glucuronoside, oestradiol-3-glucuronoside, oestriol-3-glucuronoside, oestradiol-17β-glucuronoside, oestriol-16α-glucuronoside, oestriol-17β-glucuronoside and 16α-hydroxyoestrone-16α-glucuronoside (for synthesis see [5]). None of the oestrogen glucuronosides examined in the concentration ranges 0–160 pg and 0–1600 pg/200 μl (values expressed in terms of oestrogen content of the conjugates) had a significant effect on the percentage of radioactive oestradiol bound. Some oestrogen sulphates, oestrone-3-sulphate, oestradiol-3-sulphate and oestradiol-17β-sulphate synthesised by the method of Joseph *et al.* [6], were also tested and found to have little effect on the percentage binding of oestradiol within the calibration range. These observations ruled out the possibility of measuring plasma oestrogen conjugates directly by competitive binding to rabbit uterine cytosol and indicated the necessity for hydrolysis of the conjugates before assay.

Separation of unconjugated and conjugated plasma oestrogens. It has been established that radioactive oestrogens mixed intimately with plasma can be recovered satisfactorily by ether extraction [3, 4]. Experimental proof that the oestrogen conjugates present in plasma are not extracted by this procedure was more difficult to obtain and until recently rested on evidence which was not entirely conclusive. Namely, that late pregnancy plasma which had been exhaustively extracted with ether to remove unconjugated oestrogens, could be hydrolysed

(hot acid) and then shown to contain free oestrogens measurable by competitive assay. Recently some radioactive conjugates [6,7-^{3}H]-oestradiol-17β-glucuronoside (30 Ci/mmol, 142 d.p.m./pg oestradiol; New England GmbH., Frankfurt/M., Germany) and [6,7-^{3}H]oestrone-3-sulphate (188 mCi/mmol, 1 d.p.m./pg oestrone, The Radiochemical Centre, Amersham, Bucks.) became available. Plasma samples (1 ml) to which these radioactive oestrogen conjugates were added ([6,7-^{3}H]-oestradiol-17β-glucuronoside, 4,300 d.p.m. and [6,7-^{3}H]-oestrone-3-sulphate, 3,600 d.p.m.) were submitted to the procedure for the extraction of unconjugated oestrogens. The distribution of conjugate radioactivity between the ether and aqueous phases (Table 1) was strongly in favour of the aqueous layer, thus providing direct evidence that the ether extraction effects a clean separation of endogenous free oestrogens from endogenous oestrogen conjugates.

Table 1. The partition of radioactivity associated with oestrogen conjugates during the extraction of plasma (1 ml) with ether (1 × 10 ml; 1 × 5ml)

Oestrogen conjugate added	Radioactivity in ether (d.p.m.)	Radioactivity in plasma* (d.p.m.)	Radioactivity extracted by ether (%)
[H^3]-Oestradiol-17β-glucuronoside	133	4147	3·2
	128	4152	3·1
	126	4154	3·0
[H^3]-Oestrone-3 sulphate	33	3577	0·9
	11	3599	0·3
	14	3596	0·4

*Radioactivity in plasma was calculated by difference because of the difficulty of counting plasma samples directly.

The conjugates were [6,7-^{3}H]-oestradiol-17β-glucuronoside (~ 4,300 d.p.m.) and [6,7-^{3}H]oestrone-3-sulphate (~ 3,600 d.p.m.).

Hydrolysis of plasma oestrogen conjugates. The aqueous layer remaining after ether extraction of the plasma sample was diluted with water (4 ml) and with 10 M–HCl (0·9 ml) and the tube was immersed in boiling water for 1 h. After cooling, solid NaCl (1 g) was dissolved in the hydrolysate, which was extracted with peroxide-free ether (1 × 10 ml, 1 × 5 ml). The ether extract was washed with saturated $NaHCO_3$ solution (pH 10·0) and subsequently taken to dryness. The chromatography and assay of the oestrogens was carried out as already described except that different dilutions of the recovered oestrogens were necessary, e.g. oestrone dilution 1 in 50, oestradiol 1 in 20 and oestriol 1 in 50 (v/v).

The recovery of oestrogens throughout the extraction and chromatography stages was monitored by a second addition of internal standards: oestrone (~ 8 pg, 6,000 d.p.m.), oestradiol (~ 12 pg, 9,000 d.p.m.), oestriol, (~ 50 pg, 3,500 d.p.m.) after the hydrolysis step. This procedure is not entirely satisfactory as it does not indicate losses which may have occurred during hydrolysis but the major part of the clinical study had been completed before isotope-labelled oestrogen conjugates of the required specific radioactivity became available. Addition of radioactive unconjugated oestrogens after hydrolysis gave more consistent recovery (45–73%) than addition before hydrolysis and all plasma concentrations reported were calculated on the former basis.

The recovery of labelled oestrogen conjugates added before the hydrolysis step was examined using the two tritium-labelled compounds [6,7-^{3}H]-oestradiol-17β-glucuronoside and [6,7-^{3}H]-oestrone-3-sulphate. The late pregnancy plasma used was exhaustively extracted with ether to remove unconjugated oestrogens and the radioactive oestrogen conjugates were added before hydrolysis. The extraction and chromatography of the oestrogens followed the procedure described for the measurement of plasma oestrogen conjugates and only peak column fractions were combined for the determination of recovery. For this reason, the percentage recovery of internal standards cited (Table 2) does not represent the

Table 2. The effect of hydrolyses with hot acid on the recovery of internal standards, [^{3}H]oestrogens and [^{3}H]oestrogen conjugates, from processed late pregnancy plasma

Internal standard	Time of addition	% Recovery* Oestrone	Oestradiol	Oestriol
[^{3}H]labelled oestrogens	After hydrolysis	64·0	56·0	54·6
oestrogens	After hydrolysis	51·0	57·2	50·8
oestrogens	After hydrolysis	63·0	57·2	49·4
oestrogens	After hydrolysis	73·0	62·0	45·0
[^{3}H]Oestrone -3-sulphate	Before hydrolysis	49·2	—	—
	Before hydrolysis	47·0	—	—
[^{3}H]-Oestradiol -17β-glucuronoside	Before hydrolysis	—	50·7	—
	Before hydrolysis	—	51·7	—

*Each value is the mean of duplicate determinations.

[H^2]-Labelled oestrogens added, oestrone, 6,000 d.p.m., ~ 9 pg; oestradiol, 9,000 d.p.m., ~ 12 pg; oestriol, 3,500 d.p.m. ~ 50 pg. [^{3}H]-Labelled oestrogen conjugates added, [^{3}H]oestrone-3-sulphate, 3,500 d.p.m. ~ 3,330 pg; [^{3}H]oestradiol-17β-glucuronoside, 5,000 d.p.m. ~ 35 pg. Only peak column fractions were combined for the determination of percentage recovery.

maximum recovery possible but the fraction of the amount added initially which was available for assay. The recovery of oestrogen conjugates under these conditions ranged from 47·0–51·7% but these figures may be misleading as many different endogenous oestrogen conjugates are hydrolysed simultaneously and the two compounds available even if present in pregnancy plasma are minor components.

The Reliability of the assay

Accuracy. The ability of the method to measure true values was assessed by recovery experiments in which known amounts of the oestrogens (oestrone 5 ng, oestradiol 5 ng and oestriol 10 ng) were added to buffer (1 ml, 5 experiments) and to male plasma (1 ml, 1 experiment). Procedural losses during ether extraction, chromatography and competitive binding assay were monitored by the addition of trace amounts of radioactive internal standards and when adjustment was made for these losses, the recovery from buffer was, oestrone $5{\cdot}8 \pm 0{\cdot}69$ ng; oestradiol $5{\cdot}5 \pm 0{\cdot}41$ ng and oestriol $10{\cdot}2 \pm 0{\cdot}35$ ng ($n = 5$). Recovery from male plasma was oestrone 4·1 ng (82%); oestradiol 5·4 ng (108%) and oestriol 10·0 ng (100%). Blank values from the oestrone, oestradiol and oestriol regions of an eluted Sephadex column uncharged with oestrogens were not measurably different from zero.

Sensitivity. The sensitivity of the assay relates to the smallest amount of non-radioactive steroid which can be distinguished from zero. In the present competitive binding assay the sensitivity, defined as twice the standard deviation of replicate blank determinations (n = 8) was of the order of 4 pg for oestrone, 3 pg for oestradiol and 16·5 pg for oestriol. The lowest concentration of oestrone deter mined in any plasma was 1·1 ng/ml corresponding to the measurement of 24 pg of oestrone in the assay; the corresponding values for oestradiol were 1·1 ng/ml (26 pg) and for oestriol 4·3 ng/ml (145 pg). The sensitivity of the method is thus suitable for the measurement of individual oestrogens in pregnancy plasma.

Precision. The precision of the method was determined only for the binding assay procedure by carrying out duplicate determinations on all samples processed. Precision (P) was expressed in terms defined by Snedecor [7] as $P = \sqrt{\Sigma d^2/2N}$, where Σd^2 is the sum of the squares of the differences between duplicate determinations and N is the number of paired determinations. In the range 2·0–20·4 ng/ml the precision of oestrone was ±0·049 ng (N = 15), that of oestradiol within the range 6·2–23·2 ng/ml was ±0·38 ng (N = 7) and that of oestriol within the range 5·7–26·2 ng/ml was ± 1·70 ng (N = 7).

Specificity. Specificity was achieved by the use of oestrogen-specific uterine receptor protein. Beside oestrogens no other steroid present in plasma, viz. cortisol, dehydroepiandrosterone, testosterone, progesterone etc. binds to any significant degree with the protein. Furthermore, the use of peak fractions of oestrogens separated by the column chromatography on Sephadex LH-20 rendered the assay of each oestrogen highly specific.

The measurement of unconjugated and conjugated oestrogens in late pregnancy plasma

The techniques described were applied to measure the unconjugated and conjugated oestrogen concentrations in serial plasma samples withdrawn at intervals from pregnant women between the 13–40th weeks of gestation. Changes in plasma oestrone, oestradiol and oestriol concentrations with the passage of time are shown graphically for three subjects in Figs. 2 (a and b), 3 (a and b), 4 (a and b). A salient and consistent feature of the change was the pattern of circulating oestrogen conjugates (Figs. 2a, 3a, 4a). In each subject the concentration of oestriol conjugates was very substantially higher than that of the other conjugated forms and rose progressively as the period of gestation lengthened. In contrast, the concentration of conjugated oestradiol remained at all times low. A slow increase in the conjugate forms of oestrone which was observed in each subject may reflect a rising concentration of oestrone-3-glucuronoside or oestrone-3-sulphate.

The plasma unconjugated oestrogen pattern showed much greater variety in individual subjects, oestradiol being the prominent component in most pregnancies (Figs. 2b, 3b and 4b). Nevertheless, the range of oestradiol concentrations in these apparently normal pregnancies is so wide that a single plasma oestradiol determination can have little clinical value. In the majority of pregnancies the plasma oestrone level remained low throughout gestation. The unconjugated oestriol concentration rose progressively as each pregnancy advanced and was occasionally higher than that of free oestradiol. This increase like that of oestradiol appeared to accelerate after the 35–37th week and probably reflects the growing contributions of the foetus. The concentrations of free oestrogens were at all time less than those of the conjugated forms and less than the concentration of

oestriol conjugates. The composition of circulating oestrogen conjugates to some degree resembles that found in urine, specifically in the predominence of oestriol conjugates and in the minor role of oestrone and oestradiol. The pattern of circulating unconjugated oestrogen did not in any way resemble the pattern of excreted oestrogen conjugates [8].

Plasma oestrogen and oestrogen conjugate concentrations in individual patients

The patients studied attended the out-patient clinics regularly because of a previous mishap at child-birth but each of the pregnancies studied terminated in the birth of an apparently healthy child. A comparison of two pregnancies which went normally to term (Figs. 2a, b and 3a, b):

1. *M* 22 yr, delivered a healthy male child, 3·34 kg; placenta 630 g (previous microcephalic baby);
2. *McK* 32 yr, second pregnancy, forceps delivery of a female child, 2·74 kg; placenta 580 g (previous abortion at 10/52, 9 yr previously);

shows the large difference in plasma oestradiol concentration associated with normal pregnancy. The former patient had high oestradiol concentrations throughout gestation and attained a concentration of 60 ng oestradiol/ml at 38 weeks whereas the latter patient reached 22·4 ng/ml at delivery. Both patients had similar free oestriol (~ 23 ng/ml) and ostriol conjugate (~ 200 ng/ml) concentrations at delivery but the heavier placenta and child was associated with the higher plasma oestradiol levels.

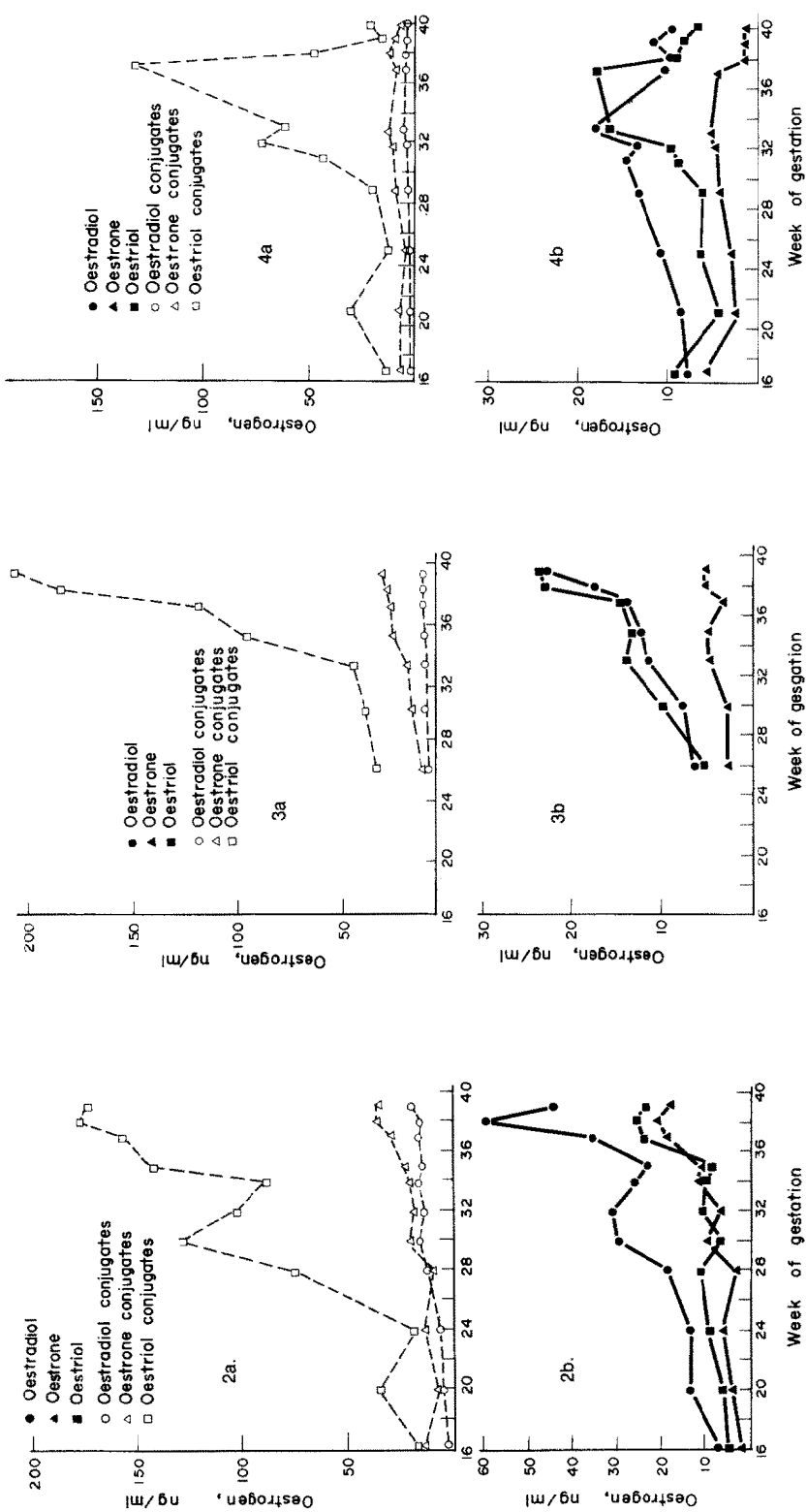
The pregnancy illustrated in Figs. 4a and b was of interest as it had to be terminated artifically because of delay at the first stage of labour.

3. *B* 28 yr, second pregnancy, delivered on 3rd June 1971 by caesarian section after induced labour of a male child, 3·9 kg; placenta 580 g.

The last plasma sample was taken on 30th April 1971 (40th week) and delivery was induced a few days later (3rd May 1971). As early as the 37th week a pronounced decrease had occurred in free plasma oestradiol concentration and the free oestriol fell substantially during the following week. The decrease in plasma oestradiol concentration preceded the dramatic fall in conjugated oestriol concentration by at least 1 week and all concentrations had fallen substantially by the 38th week, 10 days before delivery. It is reasonable to suppose that any fall in urinary oestriol, which is normally used as an indication of foetal embarrassment, would not have occurred before the 38th week.

Comparison with previous assays of plasma oestrogen concentrations

Comparison of the oestrogen plasma concentrations established in the present study with those of previous workers (Table 3) is possible only to a limited degree as the majority of assays have been conducted after hydrolysis of conjugated oestrogens. This step gives values for total oestrogens and conceals the large differences between free and conjugate forms. Earlier assays by colorimetry and fluorimetry appear to give values for oestrone which are too high while plasma free oestradiol values cited do not approach concentrations above 40 ng/ml recorded in the subject *M*. Measurements of conjugated oestrogens carried out by Touchstone and Murawec [12] by gas–liquid chromatography on a 100 ml sample are at a variance with the present results in several respects but notably in the high oestrone concentrations cited in the earlier work. Similar differences are also ob-



Figs. 2–4. The figures represent changes in the concentration of plasma oestrogens in late pregnancy. Figures 2a, 3a, 4a show changes in the concentration of oestrogen conjugates (oestradiol ○——○, oestrone △——△, oestriol □——□). Figures 2b, 3b, 4b show changes in the concentration of unconjugated oestrogens (oestradiol ●——●, oestrone ▲——▲, oestriol ■——■).

Fig. 2.

Fig. 3.

Fig. 4.

Table 3. Literature reports of plasma oestrogen concentrations in late pregnancy

Reference No.	Methods of Measurement	Sample Volume	Hydrolysis of Conjugates	Oestrogens ng/ml Oestrone	Oestradiol	Oestriol
(9)	Fluorimetry	8	+	26·5–103·0	12·5–29·3	42·8–175
(10)	Fluorimetry	10	+	13–108	0·5–15	21·6–27·0
(11)	Fluorimetry	1	+	—	—	100–550
(12)	Fluorimetry ·	100	−	14·2	8·0	3·6
	Glucuronosides		+	4·4	6·9	28·8
	Sulphates		+	41·0	15·7	4·0
(13)	Fluorimetry	5–8	+	−	−	90–200
(14)	Fluorimetry	10–50	−	−	2–23	−
(15)	Double isotope dilution	10	−	1–15	1–30	
(16)	Double isotope dilution	10	−	1–10	2–30	—
(17)	Gas chromato-	200–500	−	11·1	19·0	6·5
	graphy-mass spectrometry		+	79·2	4·4	124·0
(18)	Competitive binding	0·02–0·2	−	—	4·5–15*	—
(19)	Competitive binding	2	−	—	4·8–19·6	—
(20)	Competitive binding	0·2	+	—	—	60–210
Present paper						
	Competitive binding	1 ml	−	2·5–20·0	6·3–59·8	4·3–24·7
			+	3·2–32·2	1·1–17·2	11·8–200·0

*Mean oestradiol level in 250 pregnancies. Highest individual value at term = 50 ng/ml.

served with the results obtained by Adlercreutz and Luukkainen[17] on pooled plasma by gas chromatography-mass spectrometry. Tulchinsky and Korenman [18] recently used competitive protein binding method for the measurement of unconjugated oestradiol in pregnancy plasma. The oestradiol levels in our study are comparable with their results, the highest individual value in their series being 50 ng/ml. Similar comparison was also found with the results of Sybulski [19] who used dilute pregnancy plasma as the source of binding protein in the competitive binding assay of unconjugated oestradiol. Competitive binding method has been used by Macourt *et al.* [20] to assay total oestriol in pregnancy plasma. The results are in agreement with the sum of unconjugated and conjugated oestriol level in the present work (15·1–224·7 ng/ml), the initial low value in our series represents 16th week of pregnancy.

A commendable feature of the present method is the small plasma sample required for differential analysis and the satisfactory agreement between duplicate determinations. The accuracy of the method is acceptable and the analysis of several samples can be completed within 48h. The necessity to hydrolyse plasma oestrogen conjugates was most disappointing for not only did this step eliminate recognizable conjugate forms but it brought about the destruction of all forms of 16α-hydroxyoestrone which is recognized as a substantial urinary metabolite.

The relative merits of determination of plasma and urinary oestrogens

In terms of the effort involved there is no doubt that the analysis of plasma

oestrogen components is less difficult than that of urinary oestrogen conjugates [8]. In this contemporary study each urine analysed involved about 2 weeks of continuous effort while the differential plasma analysis of several plasma samples could be completed in duplicate within 48 h. While the plasma analysis gives the composition of the medium only at the time of sampling, the method permits serial samples collected at intervals to be analysed simultaneously in order to reveal rapid changes associated with normal and abnormal gestation. There is little doubt that the plasma reflects these changes more rapidly and faithfully especially in relation to the important components: free oestradiol and conjugated oestriol. The former component, which presumably indicates placental function is not recorded in any way by urinary analysis and the latter conjugate which reflects both foetal and placental function can be measured just as easily in plasma as in urine. The method of plasma conjugate analysis would be improved if isotope-labelled oestriol-16α-glucuronoside were available to monitor recovery throughout all stages of the assay.

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