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# AN ASSESSMENT OF THE TOTAL ESTRONE, ESTRADIOL-17$\beta$ AND ESTRIOL IN HIGH RISK PREGNANCY PLASMA*

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SUMMARY

A fluorometric method has been established for the simultaneous measurement of total estrone ($E_1$), estradiol-17$\beta$ ($E_2$) and estriol ($E_3$) using 1 ml of late pregnancy plasma. Serial determinations of the three estrogens were performed on fourteen patients in their third trimester of pregnancy. Mean $E_1$ levels ($5{\cdot}3 \pm 3{\cdot}1$ $\mu$g/100 ml plasma) were significantly higher ($P < 0{\cdot}001$) than mean $E_2$ values ($2{\cdot}9 \pm 1{\cdot}8$ $\mu$g/100 ml plasma). Excellent correlation ($P < 0{\cdot}01$) was observed between $E_1$ and $E_2$ values. Though there was a lack of correlation when either $E_1$ or $E_2$ was compared with $E_3$, good correlation was observed ($P < 0{\cdot}05$) when $E_1$ and $E_2$ together were compared with $E_3$. Estriol levels were found to be 1·8 to 12·7 times higher than the combined $E_1$ and $E_2$ values. Of the total classical estrogens, $E_1$ accounted for $15{\cdot}9 \pm 10{\cdot}2\%$, $E_2$ $8{\cdot}4 \pm 5{\cdot}7\%$ and $E_3$ $75{\cdot}7 \pm 11{\cdot}6\%$. Of the total estrone $88 \pm 5\%$ was conjugated, whereas $46 \pm 2\%$ of the total $E_2$ and $86 \pm 12\%$ of the total $E_3$ were in the conjugated form. Plasma from a severe-preeclamptic patient was found to convert [$^{14}$C]-$E_2$ to [$^{14}$C]-$E_1$, the extent of this conversion being 17% and 22% of the initial [$^{14}$C]-$E_2$ used. It is suggested that the use of $E_2$ in the management of high risk pregnancies may occasionally provide misleadingly low values, unless $E_1$ is measured as well.

## INTRODUCTION

Recent studies in our laboratory showing a correlation of plasma estriol levels with fetal well-being have resulted in the postulation of guidelines for the application of serial plasma estriol determinations in the clinical management of high risk pregnancies[1]. Measurement of plasma estradiol has also been recommended to evaluate fetal status[2–4]. For patients with Rh isoimmunization disease, Tulchinsky *et al.*[5] have suggested simultaneous measurements of $E_2$, $E_3$, and progesterone to predict fetal well-being and have reported considerable success using this approach. In our efforts to investigate and ascertain reliable parameters for the management of high risk pregnancies, it was decided to establish a simple, rapid method for the simultaneous estimation of $E_1$, $E_2$ and $E_3$ using 1 ml of late pregnancy plasma.

## EXPERIMENTAL

### *Material*

Radioactive steroids: [4-$^{14}$C]-estrone (S.A. 58 mCi/mmol) and [4-$^{14}$C]-estradiol (S.A. 58 mCi/mmol) were purchased from Amersham–Searle Corporation, Arlington Heights, Illinois. The purity of these compounds was checked on thin-layer chromatograms, using a benzene–methanol (9:1 v/v) solvent system. [6,7-$^3$H]-estriol (S.A. 2·5 Ci/mmol) was purified by thin-layer chromatography using a cyclohexane–ethyl acetate (1:1 v/v) system.

Estrone, estradiol-17$\beta$ and estriol were purchased from Steraloids, Inc., Pawling, New York. Purity was checked by melting point determination.

Instant thin-layer chromatography (t.l.c.) equipment was purchased from Gelman Instruments Company, Ann Arbor, Michigan. Separation of $E_1$ and $E_2$ was achieved on t.l.c. silica gel G sheets, using a chloroform–cyclohexane (7:3 v/v) solvent system. Areas of radioactivity on the sheets were localized on a Nuclear Chicago Actigraph III scanner.

A Turner model 430 spectrofluorometer was used to determine fluorescence.

Solvents, reagents and glassware were the same as described previously[6], with the exception of the carbonate buffer, which was prepared by dissolving 78 g NaOH and 62 g $NaHCO_3$ in 1 l of glass distilled water.

The fourteen patients included in this study were from our "high risk" pregnancy clinic. Clinical data is given in Table 1. In each case, the pregnancy resulted in a viable, mature infant.

### *Method*

The method is an extension of our previously published method[6] for the estimation of estriol in pregnancy plasma. A brief outline follows:

Hydrolysis: To plasma (1 ml) were added 3 ml glass distilled water, 0·7 ml conc. HCl, and approx. 5000 c.p.m. each of [$^{14}$C]-$E_1$, [$^{14}$C]-$E_2$ and [$^3$H]-$E_3$.

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Table 1. Clinical data on patients included in this study. In each case, the pregnancy resulted in a mature, viable infant

| Patient | Age | Parity | Clinical Impression |
|---|---|---|---|
| C.E. | 26 | G2P1A0 | Diabetes, insulin requiring |
| D.C. | 18 | G1P0A0 | Pre-eclampsia, twins |
| E.J. | 25 | G3P2A0 | Diabetes, insulin requiring |
| E.P. | 18 | G1P0A0 | Pre-eclampsia |
| E.S. | 20 | G3P2A0 | History of premature babies |
| F.W. | 27 | G1P0A0 | Hypertension |
| G.W. | 14 | G1P0A0 | Pre-eclampsia |
| I.W. | 39 | G10P6A3 | Gestational diabetes, history of stillborn |
| J.B. | 29 | G4P2A1 | Family history of diabetes |
| J.N. | 21 | G3P2A0 | Previous Cesarean sections |
| J.T. | 27 | G7P6A0 | History of premature babies |
| M.G. | 32 | G4P3A0 | Growth retardation |
| M.P. | 26 | G3P2A0 | Hypertension |
| M.S. | 34 | G8P7A0 | Hypertension |

The tubes were covered with ground glass stoppers, hydrolyzed in a boiling water bath for 40 min and then cooled.

Extraction: "Carbonate buffer" (5 ml) was added to each tube, the contents mixed and extracted once with 15 ml freshly distilled ether. The two phases were separated by centrifugation at 18000 rev./min for 2 min, the ether phase transferred to another set of tubes and the contents evaporated under nitrogen.

Isolation of $E_1$, $E_2$ and $E_3$: five ml each of glass distilled water and of a benzene–n-hexane mixture (1 : 1 v/v) prepared just before use was added to the residues, which were extracted by shaking vigorously 100 times followed by centrifugation at 1800 rev./min for 2 min. The upper organic phase containing $E_1$ and $E_2$ was transferred to a clean set of tubes. The aqueous phase (containing estriol) was re-extracted with 10 ml ether, the ether phase transferred and evaporated under nitrogen. The residue was processed as described before[6] and the amount of estriol estimated.

Organic phase: the benzene–n-hexane phase containing $E_1$ and $E_2$ was evaporated to dryness, reconstituted in 300 $\mu$l methanol and applied to t.l.c. sheets. The chromatograms were developed, allowing the solvent front to move a distance of 10 cm. from the line of application. This process took approximately 10 min, after which the sheets were removed from the chamber and air dried. Radioactive zones corresponding to $E_1$ and $E_2$, located by scanning, were cut out and the steroid eluted using 10 ml ether. The eluates were evaporated under nitrogen, the residue redissolved in 1 ml methanol and 0·1 ml aliquots taken to assay for radioactivity in order to determine procedural losses. To the remainder was added 20 mg hydroquinone in 0·2 ml ethanol and the contents evaporated under nitrogen and further processed as described for estriol[6], with the exception that the reference standard tubes contained 50 ng of either $E_1$ or $E_2$ for Kober color development. The amount of $E_1$ and $E_2$ was determined by a comparison of the degree of fluorescence in the sample tubes with that observed in the reference standard tubes.

*Comments on methodology*

*Extraction following hydrolysis.* In our previously published method for plasma estriol[6], 1·5 ml 5 N NaOH followed by 4 ml concentrated carbonate buffer pH 10·5 was used to maintain the pH of the mixture between 8–9·5. This step is therefore critical. If the proportions of the NaOH and $NaHCO_3$ solutions are not accurately delivered, the resultant pH may be higher than 9·5, thus resulting in retention of estrogens in the alkaline phase; or if lower than 8, may form an emulsion following the addition of ether. To obviate this problem and to simplify the operation, a mixture of 78 g NaOH and 62 g $NaHCO_3$/l was prepared. Five ml of this "carbonate buffer" maintains a pH of between 9–9·5.

*Isolation of $E_1$ and $E_2$.* Separation of $E_1$ and $E_2$ is satisfactorily achieved using t.l.c. sheets. The time involved in the development of the chromatograms, using a solvent front position 10 cm. ahead of the line of application, is roughly 10 min. A second set of four chromatograms can be placed in the chamber while the first lot of four is being scanned at a rate of 325 cm./h, thus requiring a total of less than 10 min to complete the operation. Elution of $E_1$ and $E_2$ from the t.l.c. sheets is performed by placing

the appropriately cut zones into glass wool plugged funnels, washing the strips with 10 ml ether, and collecting the eluate in the "Kober" tubes. The ether is dried under nitrogen, the residue redissolved in 1 ml methanol and 0·1 ml aliquot transferred to the counting vials for radioactivity assay. Single isotope $^{14}C$ channel setting was used to estimate the amount of radioactivity in the $E_1$ and $E_2$ fractions. For $E_3$, a double isotope channel setting was used to totally eliminate small amounts of [$^{14}C$]-$E_1$ and [$^{14}C$]-$E_2$ (5–7%) which may have been retained in the $E_3$ fraction. The amount of radioactivity in the tritium channel is used to calculate procedural losses for $E_3$.

*Kober color reaction.* For estimation of $E_1$ and $E_2$ in the plasma samples, 50 ng of reference standards are used in separate tubes for Kober color development along with the plasma extracts. For fluorescence assay, a solvent blank of Ittrich reagent is used to zero the fluorometer. The reference standard tubes are placed in position and the fluorometric reading set at 100. The fluorescence from the processed plasma samples is then measured and the amount of $E_1$ or $E_2$ calculated as follows:

$$\frac{S \times P_f \times 100}{S_f \times r \times 0{\cdot}9} = \mu g \text{ steroid}/100 \text{ ml plasma}$$

where $S$ = amount in the reference tubes (50 ng for $E_1$ and $E_2$ and 100 ng for $E_3$); $S_f$ = fluorescent units from reference standards; $P_f$ = observed fluorescent units from plasma samples; $r$ = % recovery of the tracer; and 0·9 = correction factor for the 1/10 aliquot taken for determination of per cent recovery. The observed fluorescence due to the "reagent blank" (tubes with 1 ml of water instead of plasma and no radioactive internal standard but processed exactly as the ones containing plasma) was not distinguishable from the Ittrich reagent blank value. Single determinations were performed on each sample. However, the analysis was repeated if the recovery of the radioactive internal standard was found to be less than 50%. Occasionally, samples from a pregnancy plasma pool were included for spot checking the accuracy of the analysis.

*Evaluation of the method used*

*Precision.* Based on 30 duplicate determinations, the standard deviation calculated as described by Snedecor[7] was 0·4 μg for $E_1$ for a range of 1·4 to 11·1 μg/100 ml plasma. The corresponding figure for $E_2$ was 0·35 μg for a range of 1·4 to 5·4 μg/100 ml plasma. The inter assay variation was 11·8% for both $E_1$ and $E_2$.

*Accuracy.* [$^{14}C$]-Estrone and [$^{14}C$]-$E_2$ were added to each of the 20 tubes containing 1 ml of plasma. The contents were then processed as described. The recovery of [$^{14}C$]-$E_1$ was 59·2 ± 5·7% and for [$^{14}C$]-$E_2$ 57·5 ± 4·8%. Additionally, when 100 ng each of $E_1$ and $E_2$ were added to a plasma pool and the aliquots processed, recovery of added $E_1$ was 96% and for $E_2$ 98%. Percent recovery of [$^{3}H$]-$E_3$ remains the same as described previously[6] i.e. 74·8 ± 2·7%.

*Sensitivity.* When 50 ng of reference standard $E_1$ or $E_2$ were used to set the fluorometer reading at 100, the smallest amount of the steroid which could be measured with 95% confidence was 5 ng. The least amount of either $E_1$ or $E_2$ which could be accurately measured (CV < 12%) when added to non-pregnancy plasma was 8 ng. Corresponding value for $E_3$ remains the same[6] i.e. 1·3 μg/100 ml plasma.

*Specificity.* Separation of $E_1$ from $E_2$ on t.l.c. sheets afforded satisfactory specificity. Further purification of each steroid fraction on thin-layer chromatograms yielded comparable results. Additionally, the fluorescence spectra of the Kober complex of the plasma samples were found to be identical to those of the reference standard steroids.

Estimation of unconjugated $E_1$ and $E_2$ was performed by our previously published method for $E_3$, utilizing a single 10 ml water-saturated ether extraction for the separation of the unconjugated form from the conjugated estrogen fraction.

*Incubation technique*

Approximately 5000 c.p.m. of purified [$^{14}C$]-$E_2$ dissolved in ethanol were added, in duplicate, to ground glass stoppered tubes. The contents were evaporated to dryness and 0·5 ml freshly obtained pregnancy plasma was added to each tube. In another tube 0·5 ml distilled water instead of plasma was added to serve as the blank. The contents in the tubes were mixed in a Vortex mixer and allowed to stand at room temperature for 3 h. Following this, the mixture was extracted, the extracted material processed as described and zones corresponding in chromatographic mobility to $E_2$ and $E_1$ were cut out, eluted and aliquots taken for radioactivity assay. The radioactive material in the [$^{14}C$]-$E_1$ zone was further purified on thin-layer chromatogram along with a mixture of authentic $E_1$ and $E_2$. Following development, the estrogen zones were localized using Folin–Ciocalteu reagent and the radioactive area determined by scanning. Additionally, separate chromatograms carrying a mixture of authentic $E_1$ and $E_2$ and either [$^{14}C$]-$E_1$ or [$^{14}C$]-$E_2$ were developed and $E_1$ and $E_2$ zones eluted and amount of radioactivity in each fraction determined.

## RESULTS

The levels of total $E_1$, $E_2$ and $E_3$ in late pregnancy plasma are shown in Table 2. The coefficient of correlation between $E_1$ and $E_2$ was highly significant (Table 3). However, no significant correlation was observed when either $E_1$ or $E_2$ values were compared with $E_3$. The sums of $E_1$ and $E_2$ when

Table 2. Amounts ($\mu$g/100 ml plasma) of estrone, estradiol-17$\beta$ and estriol in third trimester pregnancy plasma

| | Amount | | | |
|---|---|---|---|---|
| Patient | $E_1$ | $E_2$ | $E_3$ | $E_3/E_1+E_2+E_3$ |
| C.E. | 7.4±3.6 | 5.4±1.6 | 32.0±12.2 | 2.8±0.8 |
| D.C. | 6.6±2.2 | 3.7±1.5 | 73.0±16.0 | 6.7±1.2 |
| E.J. | 7.4±3.0 | 4.2±1.7 | 19.4±8.8 | 1.8±0.5 |
| E.P. | 4.0±1.4 | 3.0±1.2 | 36.1±17.6 | 5.2±2.1 |
| E.S. | 2.0±1.1 | 3.8±0.9 | 30.8±15.4 | 5.9±4.8 |
| F.W. | 2.8±1.5 | 2.3±1.4 | 38.9±6.0 | 8.9±3.9 |
| G.W. | 2.3±0.8 | 2.1±1.0 | 47.7±21.4 | 11.3±4.3 |
| I.W. | 1.4±0.9 | 0.9±0.6 | 21.3±7.0 | 12.7±5.5 |
| J.B. | 5.2±1.3 | 2.7±1.7 | 26.0±13.3 | 4.2±1.9 |
| J.N. | 4.9±1.9 | 1.5±0.2 | 28.3±3.9 | 4.7±1.6 |
| J.T. | 4.8±2.7 | 2.0±0.8 | 21.9±3.4 | 4.8±3.7 |
| M.G. | 4.0±2.6 | 1.4±1.1 | 16.9±6.8 | 3.5±1.5 |
| M.P. | 5.8±3.7 | 3.7±1.2 | 22.5±12.2 | 2.7±2.5 |
| M.S. | 8.6±3.2 | 0.7±0.4 | 35.5±20.8 | 3.6±1.2 |
| M±S.D. (n=89) | 5.3±3.1 | 2.9±1.8 | 31.7±19.5 | 5.1±3.8 |
| % of total $E_1+E_2+E_3$ | 15.9±1.02 | 8.4±5.7 | 75.7±11.6 | |

Normal values (M±S.D.) obtained for third trimester pregnancy are:

$E_1$=4.4±2.3 (n=34)

$E_2$=2.9±1.3 (n=25)

$E_3^*$=21.6±9.7 (n=187)

*taken from Mathur et al[8]

Table 3.

Observed correlation coefficient (r)

| Item | r* | Significance |
|---|---|---|
| $E_1$ versus $E_2$ | 0.319 | p<0.01 |
| $E_1$ versus $E_3$ | 0.186 | N.S. |
| $E_2$ versus $E_3$ | 0.189 | N.S. |
| $E_1$ + $E_2$ versus $E_3$ | 0.233 | p<0.05 |

* 87 degrees of freedom

compared with $E_3$ showed good correlation. The proportions of unconjugated and conjugated estrogens in a pregnancy plasma pool are given in Table 4. Levels of these classical estrogens were measured in plasma obtained from a patient whose pregnancy was complicated by preeclampsia and diabetes (Table 5). In another patient with preeclampsia, blood was drawn for eight consecutive days and $E_1$ and $E_2$ measured (Table 6).

*Incubation studies*

Incubation of [$^{14}$C]-$E_2$ with 0·5 ml freshly obtained plasma from a severely preeclamptic patient resulted in 17% and 22% (duplicate) of the initial radioactivity in the $E_1$ zone. The radioactive material in the $E_1$ zone thus obtained coincided with authentic $E_1$ stain when further developed on a thin-layer chromatogram. No radioactive material was found in the $E_1$ zone when water, instead of

Table4.

Relative amounts of 'free' and conjugated estrogens in late pregnancy plasma pool.

| Estrogen | Amount (ug%) | % of Total |
|---|---|---|
| Estrone: | | |
| free | 0.4±0.2 | 11.7±4.7 |
| conjugate | 3.3±0.4 | 88.3±4.7 |
| Estradiol: | | |
| free | 1.5±0.2 | 54.3±2.0 |
| conjugate | 1.2±0.2 | 45.7±2.0 |
| Estriol:* | | |
| free | 3.1±0.9 | 14.1±4.9 |
| conjugate | 20.0±7.4 | 85.9±5.2 |

* Taken from Mathur et al (8)

## DISCUSSION

The purpose of this investigation was to establish a relatively simple chemical method for the simultaneous measurement of total $E_1$, $E_2$ and $E_3$ using only 1 ml of pregnancy plasma.

The method outlined, which is an extension of our previously published method for $E_3$ in pregnancy plasma[6] has been evaluated by the standard criteria, i.e., precision, accuracy, sensitivity and specificity. One trained technician can process six samples, in duplicate, in an eight hour working day. The fact that the method utilizes only 1 ml of plasma as opposed to much larger volumes required by other methods[10–12] is an asset. For clinical management of high risk pregnancies, estimation of estriol in plasma is extensively used at our institution[1]. Thus, no additional amount of plasma was used as blank. Also, no radioactive material was obtained in $E_1$ zone if only [$^{14}$C]-$E_2$ was spotted on t.l.c. and vice versa. No conversion of [$^{14}$C]-$E_2$ to [$^{14}$C]-$E_1$ was obtained when plasma from five other high risk pregnancy patients selected at random including two preeclamptics was used.

Table 5. Amount (μg/100 ml plasma) of estrone, estradiol and estriol in a patient with preeclampsia and diabetes

| | $E_1$ | | $E_2$ | | | |
|---|---|---|---|---|---|---|
| Weeks gestation | %Rec.of Int.Std. | Amount | %Rec.of Int.Std. | Amount | $E_1$+$E_2$ Amount | $E_3$ Amount |
| 34 | 62.8 | 6.7 | 59.6 | 0.3 | 7.0 | 10.6 |
| 36 | 69.2 | 4.9 | 64.2 | 3.9 | 8.8 | 7.4 |
| 38 | 56.1 | 3.6 | 51.7 | 4.0 | 7.6 | 36.9 |
| 40 | 62.7 | 5.4 | 54.4 | 2.5 | 7.9 | 19.1 |
| M±S.D. | 62.7±5.3 | 5.2±1.3 | 57.5±5.6 | 2.7±1.7 | 7.8±0.8 | 26.0±13.3 |
| CV | 8.4% | 25.0% | 9.7% | 63.0% | 10.2% | 51.2% |

Table 6. Amounts (μg/100 ml plasma) of the classical estrogens in a preeclamptic patient during 38–39th week of gestation

| | $E_1$ | | $E_2$ | | | |
|---|---|---|---|---|---|---|
| Date | %Rec. Int.Std. | Amount | %Rec. Int.Std. | Amount | $E_1$+$E_2$ Amount | $E_3$ Amount |
| Jan. 23 | 63.5 | 5.7 | 56.5 | 6.3 | 12.0 | 86.0 |
| 24 | 61.7 | 6.0 | 59.9 | 4.4 | 10.4 | 64.6 |
| 25 | 68.9 | 5.2 | 61.9 | 4.4 | 9.6 | 46.2 |
| 26 | 63.2 | 8.3 | 69.1 | 3.2 | 11.5 | 80.5 |
| 27 | 71.3 | 7.0 | 69.3 | 1.9 | 8.9 | 76.5 |
| 28 | 64.2 | 5.9 | 58.2 | 5.5 | 11.4 | 58.2 |
| 29 | 65.2 | 10.8 | 58.3 | 2.6 | 13.4 | 97.4 |
| 30 | 66.5 | 7.9 | 64.6 | 2.7 | 10.6 | 79.9 |
| M±S.D. | 65.6±3.2 | 7.1±1.9 | 62.2±5.0 | 3.9±1.5 | 11.0±1.4 | 73.7±16.4 |
| CV | 4.9% | 26.7% | 8.0% | 38.4% | 12.8% | 22.2% |

plasma is required to determine $E_1$ and $E_2$ for investigative purposes.

For the management of pregnancies complicated by Rh-isoimmunization disease, Tulchinsky *et al.*[5] have recently suggested use of plasma $E_2$, $E_3$ and progesterone and have reported considerable success using ratios of $E_2/E_3$ and $P/E_3$ to predict

fetal status. Thus the proposed method can be used by clinical laboratories for simultaneous estimation of the classical estrogens in fetal monitoring.

The amounts of total $E_1$ and $E_2$ observed (Table 2) in this study are in agreement with values reported by other investigators for uncomplicated late pregnancies [10, 12, 13]. Amounts of $E_3$ observed are in agreement with values previously published by us [1, 6], Nachtigall and associates [14, 15], using fluorometric techniques, and by Macourt *et al.* [16], using a competitive protein binding method for total $E_3$.

The proportions of the individual estrogen to the total of the three classical estrogens are in agreement with the values reported by Smith [17]. Our results as regards the proportions of the unconjugated and conjugated forms of $E_1$ and $E_2$ (Table 4) are also in general agreement with the previously published reports [12, 17].

Unconjugated $E_3$ measured by this procedure accounted for 14% of the total $E_3$ in the third trimester of pregnancy [8]. This is in agreement with the recent report of Goebelsman *et al.* [9] who included a chromatographic step and found that 13·8% of the total $E_3$ was in the unconjugated form.

The main site of estrogen synthesis in late pregnancy is the placenta. The placenta utilizes dehydroepiandrosterone sulfate (DHAS) of maternal and fetal origin for the synthesis of $E_1$ and $E_2$. Of the total $E_1$ and $E_2$ synthesized by the placenta, about half is derived from the maternal precursor and the other half from fetal DHAS [18]. Since the placenta cannot convert either $E_1$ or $E_2$ to estriol because it lacks the 16$\alpha$-hydroxylase enzyme system (for a recent review see [19]), it aromatizes 16$\alpha$-HO-DHAS (mainly of fetal origin) to yield $E_3$. This, the so called neutral pathway of $E_3$ synthesis [20] does not involve either $E_1$ or $E_2$ as intermediates. In our study highly significant correlation was observed between $E_1$ and $E_2$ levels (Table 3), which is due to this mode of synthesis and the rapid equilibrium established between $E_1$ and $E_2$ in the maternal liver [21]. Diczfalusy and his associates [22, 23] have shown that the placenta releases more $E_1$ than $E_2$ at least into the fetal circulation. Additionally, $E_2$ reaching the maternal liver is rapidly metabolized and converted mainly to $E_1$ [24]. Thus, higher levels of $E_1$ than $E_2$ ($P < 0{\cdot}001$) are found in the maternal circulation. No significant correlation was observed when either $E_1$ or $E_2$ values were compared individually with $E_3$ levels, substantiating an earlier report [2]. However, good correlation was found when $E_1$ plus $E_2$ was compared with $E_3$ levels (Table 3).

In a patient whose pregnancy was complicated by pre-eclampsia and diabetes (Table 5), $E_1$ and $E_2$ were measured from 34th week of gestation. Marked variations were observed in $E_1$ and $E_2$ levels. However, these variations were reduced (CV = 10·2%) when the sums of $E_1$ and $E_2$ were taken into consideration. Since the interassay variation of our method is 12%, it is concluded that the $E_1$ plus $E_2$ levels remained constant from the 34th until the 40th week of gestation, which is in agreement with observations of Sybulski and Maughan [4] as regards $E_2$. If this pregnancy had been monitored by plasma $E_2$ values alone, a level of 0·3 $\mu$g% at 34th week would have suggested fetal jeopardy. That this was not the case is indicated by $E_3$ level of 10·6 $\mu$g% [1]. The variation in plasma $E_2$ values could not have been due to the lack of proper separation of $E_1$ from $E_2$ since the recoveries of the radioactive internal standards were within our normal range. To further investigate the variation observed, blood samples were drawn daily for eight days from a patient with pre-eclampsia and estogen determinations were performed (Table 6). The coefficient of variation (CV) for $E_1$ and $E_2$ was 26·7% and 38·4% respectively. The CV for the sums of $E_1$ and $E_2$ was, however, only 12·8% which is slightly higher than the inherent error of our method. Thus, very little, if any, change had occurred in the $E_1$ plus $E_2$ levels of this patient for the eight day period she was studied. In contradistinction, $E_2$ levels progressively dropped from 6·3 $\mu$g% on day 1 to 1·9 $\mu$g% on day 5—a drop of approximately 70%—suggesting fetal distress, an interpretation not supported by the high $E_3$ levels and clinical evaluation of the patient, who subsequently delivered healthy twins.

Use of plasma $E_2$ in the clinical management of pregnancy has been suggested [2–4]. In a recent study Townsley *et al.* [2] evaluated usefulness of various parameters in the management of high risk pregnancies and observed a 29% incidence of misleading low $E_2$ levels. The time lag between getting a blood sample from a patient and its receipt in the laboratory may at times involve several hours. Our observations of variable $E_2$ levels led us to two possible explanations: either the $E_2$ levels do fluctuate widely in high risk pregnancies, or $E_2$ may be further metabolized, including its conversion to $E_1$ after the sample was drawn. The first explanation is not very probable in view of the fact that CV for $E_1$ was considerably less than that for $E_2$. Release of these two steroids from the placenta and further equilibrium established in the maternal liver cells would tend to rule out the possibility of fluctuations of this magnitude only in $E_2$ without causing similar changes in circulating levels of $E_1$.

Our observation that plasma from a severe preeclamptic patient converted [$^{14}$C]-$E_2$ to [$^{14}$C]-$E_1$ may raise doubts about the usefulness of $E_2$ determination in the management of high risk pregnancies. However, no such conversion was observed when plasma from five other high risk pregnancies randomly selected, including two with pre-eclampsia, was used. Thus, it is possible that in some complicated pregnancies further conversion of $E_2$ to $E_1$ may still proceed after the samples are drawn. The placenta is a rich source of the $E_2$-17$\beta$-

hydroxy-dehydrogenase enzyme system involved in the conversion of $E_2$ to $E_1$. Recently, Tulchinsky *et al.*[25] have observed this conversion in samples obtained from the retroplacental space, suggesting the presence of this enzyme. It is not inconceivable that in some complicated pregnancies the enzyme may be released into the maternal circulation in amounts sufficient to cause changes in $E_2$ levels. In this context, it must be noted that the placenta is reported to synthesize certain enzymes, such as renin, diamine-oxidase, heat stable alkaline phosphatase and oxytocinase[19] and releases them to the maternal organism.

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