The Plasma Estradiol as an
Index of Fetoplacental Function

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ABSTRACT A radioligand assay has been developed for the measurement of unconjugated 17β-estradiol in as little as 0.01 ml of pregnancy plasma employing rabbit uterine cytosol as specific binder and activated charcoal as nonspecific adsorbant. Large numbers of samples could be processed simultaneously at relatively little expense and results were obtainable within 3 hr. The procedure was sensitive to 1 ng/ml. No diurnal or positional variations were found. Values from 250 unselected normal patients showed a constantly rising mean plasma E2 from 18 to 35 wk gestation from 4.5 to 14 ng/ml. From 35 to 40 wk, mean E2 rose only to 15 ng/ml and the range of values increased substantially. When 22 normal pregnant subjects were followed serially, rising levels of plasma E2 were found with no significant fall ever seen. By contrast, patients exhibiting fetal distress generally had falling or reduced E2 levels. However, 3 cases of Rh isoimmunization had elevated or normal E2 concentrations even after clearcut evidence of fetal demise. A decrease of 45% in E2 concentration was associated with intraamniotic instillation of hypertonic saline prior to delivery supporting the view that placental conversion of maternal adrenal precursors is responsible for about half of the E2 production in pregnancy. The postpartum clearance of endogenous E2 was measured and found to fit a two compartment model with mean half-time of 22 min and 7 hr. Follicular phase levels of E2 were attained by 35 hr postpartum. The concentration of unconjugated E2 in pregnancy plasma correlated as well with the state of the placenta as other placental hormone measurements and holds promise of being a rapid, inexpensive, and reliable method of following patients with high-risk pregnancies in a variety of clinical settings.

INTRODUCTION

Since the discovery of Spielman, Goldberger, and Frank in 1933 (1) that, upon the death of the fetus, urinary bioassayable estrogens decrease markedly, it has been clear that this phenomenon might have clinical applications in assessing fetoplacental function.

Subsequently, with the development of steroid measurement methods, estimation of the urinary estriol (E3) has been used extensively for assisting the obstetrician in the diagnosis of fetal distress. Aside from technological problems which have been gradually overcome, urinary E3 assay has intrinsic disadvantages. The need to collect urine results in errors if there are incomplete 24 hr collections. Because of dependance on hepatic conjugation and renal clearance wide daily variations in urinary E3 excretion have been described (2-6), increasing the complexity of clinical interpretation.

Measurement of the plasma E3 (6, 7) has not attained widespread acceptance, perhaps because of technical difficulties, but results may be obtained within a single day. A search for other means of evaluating placental function has been carried out recently. Plasma levels of various hormones (8, 9), blood components (10), and enzymes (11, 12) have been suggested as criteria of assessing placental function and some of these techniques such as blood human chorionic somatomammotropin (placental lactogen) termed (HCS) (9) are promising.

Development in our laboratories of a radioligand assay method for the measurement of unconjugated 17β-estradiol (E2) (13-15) and estrone (E1) (16) in non-

1 Abbreviations used in this paper: E2, Estrone—Estra-1,3,5(10)-triene-17-one,3-hydroxy; E3, 17β-Estradiol—Estra-1,3,5(10)-triene-3,17β-diol; E4, Estriol—Estra-1,3,5(10)-triene-3,16α,17β-triol; HCG, human chorionic gonadotropin; HCS, human chorionic somatomammotropin (placental lactogen).
pregnant individuals led us to explore the possibility of measuring E2 in pregnancy plasma and testing its effectiveness as a predictor of fetal distress.

We now report an extremely simple, inexpensive, and rapid technique for the radioligand assay of unconjugated plasma E2 in pregnancy. A single individual can reliably measure the concentration of E2 in large numbers of plasmas in 3 hr using minute amounts of blood.

Over 250 sequential and individual estrogen determinations have revealed the pattern of change during normal pregnancy and have demonstrated the alterations in 16 cases of fetal distress. Changes in the plasma E2 resulting from induction of abortion using hypertonic saline infusion have been characterized. The pattern of decline of endogenous E2 from the time of parturition has been shown.

On the basis of these data it is suggested that measurement of E2 during pregnancy by the radioligand assay procedure may provide considerable physiological information and may be of significant clinical applicability, particularly in the early diagnosis of placental insufficiency.

**METHODS**

6,7-3H-Estradiol, 42.0 Ci/m mole, obtained from New England Nuclear Corporation (Boston, Mass.) was used without further purification for about 1 month and discarded. Spectroquality ethylene glycol and activated charcoal (Norite A) were obtained from Matheson, Coleman, and Bell (Cincinnati, Ohio); dextran D grade was obtained from Mann Research Laboratories Inc., New York. Biosolve-BBS® was obtained from Beckman Instruments, Fullerton, Calif. All other chemicals were reagent grade.

The subjects were all volunteers who gave informed consent. For the postpartum studies, the subjects were housed in the Harbor General Hospital Clinical Research Center.

**Assay Procedure.** The specific estrogen binder was prepared from immature rabbit uteri as previously described (13-15). Fresh or freshly frozen uteri from immature rabbits were obtained from a commercial slaughterhouse. Uterine estrogen binder has been prepared successfully from rat, calf, horse, and human uterus (15) as well as rabbit. Uteri were homogenized in 3 volumes of a buffer consisting of 0.01 M Tris-HCl pH 8.0, 0.001 M EDTA, 0.25 M sucrose, at 4°C in the Waring Blender using four 30-sec pulses and cooling intervals of 2 min. The homogenate was centrifuged at 1000 g for 15 min and the supernatant centrifuged at 105,000 g for 90 min at 4°C. This supernatant (cytosol), stored in liquid N2 in small portions, was stable as a potent estrogen binder for months.

The E2 assay was carried out in 10 × 75 mm disposable glass centrifuge tubes. Radioactive E2 and standard E2 solutions, were made up in assay buffer consisting of 8 volumes 0.01 M Tris-HCl pH 8.0, 0.001 M EDTA, and 2 volumes of ethylene glycol. To a final vol of 0.5 ml were added either blank or various concentrations of unknown 9,10-3H-Estradiol. Standards were added to a total of 0.5 ml of assay buffer to a total of 0.5 ml of assay buffer to a final of 0.5 ml.

The standard curves were used to determine the concentration of the unknown E2 fraction present. Standard curves were prepared by adding 0.5 ml of a suspension containing 0.5% activated charcoal and 0.05% dextran to 0.01 M Tris-HCl pH 8.0. After incubation for 15 min in an ice bath, samples were centrifuged for 10 min at 4°C and the supernatants decanted into counting vials containing 10 ml of scintillation solution consisting of 160 ml Biosolve, 42 ml Liquidor and toluene to 1 liter total volume. The samples were counted to a total of 5000 counts at an efficiency of 35%.

**TABLE I**

<table>
<thead>
<tr>
<th>Ratio of association constants</th>
<th>16 hr 0°C</th>
<th>2 hr 23°C</th>
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</thead>
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<tr>
<td>Estrone</td>
<td>0.41</td>
<td>0.08</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.10</td>
<td>0.05</td>
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</table>

Unradioactive estrone and estriol in increasing concentrations were allowed to compete with 1H-labeled estradiol for binding sites in uterine cytosol at 0°C for 16 hr and at 23°C for 2 hr.

From the competition curves, employing previously described procedures (19), the ratio of association constants (RAC) were calculated.

The protein-bound and free 3H-labeled E2 were separated by adding 0.5 ml of a suspension containing 0.5% charcoal and 0.05% dextran in 0.01 M Tris-HCl pH 8.0. After incubation for 15 min in an ice bath, samples were centrifuged for 10 min at 4°C and the supernatants decanted into counting vials containing 10 ml of scintillation solution consisting of 160 ml Biosolve, 42 ml Liquidor and toluene to 1 liter total volume. The samples were counted to a total of 5000 counts at an efficiency of 35%.

**Validation of the assay system.** As previously described (14, 15) standards ranged from 10 to 200 pg with 20 pg always > 2 so different from 0. A typical standard curve has been presented (14). For unknowns run at 0.01 ml, then, the sensitivity of the assay was 2 ng/ml. We found that the use of 0.020 ml of plasma did in no way alter the assay so that under those circumstances a sensitivity of 1 ng/ml was attained. Furthermore, dried ether extracts of 0.2 ml of plasma or less also gave identical plasma concentration values at a sensitivity of 0.1 ng/ml. Blanks using nonpregnancy plasma gave values indistinguishable from 0.

The precision of the assay system has been previously evaluated (14) with an index of precision λ of the standard curve of 0.06. A number of studies have been carried out to validate the specificity of the estradiol assay. Specificity of the uterine receptor for estrogens has been demonstrated (13, 17). Comparability of estrogen measurements with those of Baird and Guevara, using a double isotope derivative procedure (18), has supported the validity of radioligand assay.

In the case of pregnancy plasma, conditions were required which could minimize the contribution of the E3, E1, and estrogen conjugates present. Accordingly, conditions were employed in which the ratio of association constants (19) of E2 and estradiol relative to E1 were low (Table 1).

In contrast to overnight incubation at 0°C, after incubation at 23°C for 2 hr, E1 and E3 had affinities only 8 and 5% that of E2 for the uterine receptor. It has been our experience that estrogen conjugates do not bind to the uterine cytosol receptor. However, to demonstrate the lack of influence of any component of pregnancy plasma on the plasma E2 determination, we have carried out a comparison of the above procedure with replicates assays as previously described (13-15). Other estrogens and estrogen conjugates were separated from the E1 fraction by ether extraction and
Samples of pregnancy plasma were assayed by the simple and the more complex radioligand assay procedures described in the text and the values obtained compared. The mean E2 was 9.45 ng/ml on direct assay and 9.65 ng/ml for extracted and chromatographed plasma.

Table II

<table>
<thead>
<tr>
<th>Direct</th>
<th>Processed</th>
<th>Direct</th>
<th>Processed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng/ml</td>
<td>ng/ml</td>
<td>ng/ml</td>
<td>ng/ml</td>
</tr>
<tr>
<td>2.0</td>
<td>2.6</td>
<td>11.6</td>
<td>13.8</td>
</tr>
<tr>
<td>15.0</td>
<td>14.9</td>
<td>6.6</td>
<td>4.6</td>
</tr>
<tr>
<td>20.0</td>
<td>21.0</td>
<td>8.8</td>
<td>5.8</td>
</tr>
<tr>
<td>5.6</td>
<td>4.6</td>
<td>7.4</td>
<td>9.0</td>
</tr>
<tr>
<td>1.1</td>
<td>0.6</td>
<td>14.0</td>
<td>16.9</td>
</tr>
<tr>
<td>14.0</td>
<td>13.2</td>
<td>18.8</td>
<td>19.5</td>
</tr>
<tr>
<td>13.0</td>
<td>12.2</td>
<td>5.4</td>
<td>4.3</td>
</tr>
<tr>
<td>6.8</td>
<td>6.3</td>
<td>18.0</td>
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<td>3.7</td>
<td>2.3</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>10.8</td>
<td>12.1</td>
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<tr>
<td>8.8</td>
<td>7.1</td>
<td>4.1</td>
<td>6.2</td>
</tr>
<tr>
<td>6.6</td>
<td>5.1</td>
<td>9.4</td>
<td>11.6</td>
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The effect of short-term postural changes on blood E2 were checked in patients from whom blood was drawn after 30 min of stabilization in each given position. There was no difference from one position to another.

Blood was drawn from 250 unselected patients visiting the prenatal clinic. Their plasma E2 values are shown in Table II. The mean E2 concentration increased gradually until the 35th wk of pregnancy when it leveled off. The scatter of individual values increased with advancing pregnancy and was larger for values above the mean than for values below it, giving a skewed distribution. As a result, 2 SD of the mean represented by the upper and lower limits, were expressed for each group separately. In the last 8 wk of pregnancy, not a single value was found to fall below 5.5 ng/ml. Twin pregnancies resulted in high E2 values.

Abnormal pregnancies

In this group of patients are those admitted for imminent fetal demise, intrauterine growth retardation or...
a condition suspected of leading to fetal wastage. The problem was identified at various stages of gestation and when possible, multiple plasma E2 determinations were carried out. In Fig. 3 the plasma E2 concentrations in each individual case are shown. Brief clinical descriptions follow.

Patient 1 was an 18 yr old, primigravida with severe preeclampsia including 4+ proteinuria and a blood pressure of 200/120. Administration of hydralazine resulted in a rapid return of her blood pressure to normal. Her plasma E2 fell sharply to subnormal levels. 1 day later fetal movement was noted to have ceased and intrauterine fetal demise was confirmed.

Patient 2 was a 33 yr old, gravida 2 para 0, with a history of 10 yr of hypertension. She was admitted for preeclampsia with 3+ proteinuria and a blood pressure of 220/130. Initial and subsequent plasma E2 concentrations were all subnormal. A further drop was noted in association with fetal death.

Patient 3. See patient Rh3 in next section.

Patients 4, 5, 6, 7, and 10 represent five patients with intrauterine fetal demise of unknown cause. All apparently had a normal gestation and none had any previous abnormal pregnancies. Of the five, patient 7, the only one in labor, had a normal plasma E2 level. She had noticed sudden cessation of fetal movement 5 days before admission and the placenta on delivery was found to be intact.

Patient 8 was a 24 yr old, gravida 2 para 1, who was thought to have intrauterine growth retardation. Her initial E2 value was considered to be borderline. She was delivered of a 5 lb. 2 oz., alive, dysmature baby via emergency cesarean section, when fetal movements ceased.

FIGURE 2 Serial plasma estradiol determinations in 22 pregnant patients. The lines connect determinations in an individual.

FIGURE 3 The plasma estradiol concentration in 16 abnormal pregnancies. The black squares represent samples obtained after a clinical diagnosis of fetal demise. Lines connect sequential determinations in an individual. Arrows indicate time of delivery.

Patient 9 was a 28 yr old, gravida 3, para 2, admitted in labor with a history of two previous cesarean sections due to cephalopelvic disproportion. A repeat cesarean section resulted in a delivery of a 4 lb. 12 oz., dysmature, alive fetus and the placenta on examination showed multiple infarcts covering 50% of the placental surface. This patient had a normal E2 concentration.

Patient 11 was a 23 yr old, primigravida who was hospitalized for preeclampsia with 1+ proteinuria and a blood pressure of 180/100. She was maintained on hydralazine and diuretics. Her plasma E2 concentration fell substantially to below normal values under observation. 1 day after the last determination, fetal heart tones were no longer heard and she was delivered of an alive infant by cesarean section.

Patient 12 was a 24 yr old, primigravida, admitted for preeclampsia with 1+ proteinuria and a blood pressure of 160/100 which was controlled by hydralazine. Fetal heart tones which were present on admission became inaudible 2 days later. Her low initial plasma E2 fell still further at the time of fetal demise.

Patient 13 was a 30 yr old, gravida 5, para 4 heroin addict who presented at 38 wk gestation with severe malnutrition and a hematocrit of 6%. She was transfused and treated with promazine hydrochloride. Fetal heart tones, which were present on admission, were noted to have disappeared 10 hr later when the patient spontaneously commenced labor. She was delivered of a 3150 g nonmacerated stillborn. The placenta was small (230 g) and contained multiple infarctions. Her plasma E2 was subnormal.

The Plasma Estradiol as an Index of Fetalplacental Function
Measurement of the plasma E₂ would have been of assistance in predicting imminent fetal demise in cases 1, 2, 11, 12, 13, and 14. In patients 4, 5, and 6, the clinical presentation was too late for fetal salvage. The value in patient 7, was clearly misleading and in 9, a normal plasma E₂ concentration failed to reveal imminent fetal demise or placental infarction. In the three cases of Rh isoimmunization disease, E₂ levels were elevated or normal even in the face of fetal demise.

Induced abortions

The disappearance rate of E₂ was determined in nine patients at from 14 to 20 wk gestation who underwent therapeutic abortion by the infusion of hypertonic saline intraamniotically for 10 min. Blood was obtained before the procedure and at the times indicated in Fig. 4. The mean initial plasma E₂ was 4.0 ng/ml. By 6 hr, 6 of the patients had attained their maximum fall. At 20 hr, before delivery, the mean value had fallen only to 2.2 ng/ml despite the fact that delivery of a stillborn fetus ensued shortly thereafter. The low values of E₂ occurred in a 22 yr old gravida, 2 para 1 with scleroderma, who had been receiving 20 mg of prednisone throughout

Patient 14 was a 22 yr old, primigravida, admitted at 26-27 wk of gestation for preeclampsia and fetal growth retardation. She had 3+ proteinuria and a blood pressure of 160/100. The uterus was the size of a 22-wk gestation and fetal heart tones were present. Her blood pressure was controlled with hydralazine over the next 4 wk but no increase in uterine size was noted. Fetal movements and then fetal heart tones ceased and the patient was delivered of an 1100 g macerated fetus by the instillation of hypertonic glucose intraamniotically. The placenta weighed 250 g and had multiple infarcts. The plasma E₂ hovered at the lower limit of normal for a 26 wk gestation and fell before fetal demise.

Patients with Rh isoimmunization disease

Patient Rh1 was a 26 yr old, gravida 3, para 2. Her plasma E₂ was abnormally high for her stage of gestation and continued to be elevated after fetal death.

Patient Rh2 was a 23 yr old, gravida 3, para 2, whose severe Rh isoimmunization disease necessitated intraventricular fetal transfusion. Fetal death ensued and she went into spontaneous labor 1 day later. Her E₂ levels, initially elevated, fell only to the normal range after fetal demise.

Patient Rh3 was a 22 yr old, gravida 3, para 2, who presented at 30 wk of gestation with evidence of intraventricular fetal demise. Her initial E₂ was in the normal range and although it fell significantly 1 wk later it remained in the normal range despite unequivocal evidence for fetal demise.

D. Tulchinsky and S. G. Korenman
pregnancy. Her initial value of 800 pg/ml fell to 200 pg/ml, a value in the nonpregnant range.

Postpartum E₂ clearance

In order to study the disappearance rate of E₂ from the blood, we selected four volunteers who had had an uneventful labor and delivery, and who had not received lactation suppressants. The initial blood sample was drawn upon delivery of the baby's head and subsequently in frequency ranging from 10 min in the first postpartum hr to 6 hr on the 3rd postpartum day. During the first 24 hr, blood was drawn through a heparinized catheter which was removed thereafter and the total amount of blood obtained from each patient did not exceed 75 ml. From three other patients, blood samples were drawn in the 1st postpartum hr only. Sensitivity of the assay system was adapted to the anticipated concentration of E₂. After 3 hr each sample was analyzed by the method for nonpregnant individuals previously described.

The results for the 1st hr are depicted in Fig. 5. The data are presented as per cent of the initial concentration. The mean initial half-life of the hormone was 22 min. Four of the seven had initial E₂ half-lives of 13 min and the other three 27, 30, and 45 min respectively. Subsequent determinations are presented in Fig. 6. A second exponential decay with half-lives of 6, 6, 6, and 8 hr was apparent. Normal early follicular phase values for E₂ of under 100 pg/ml were reached in each case by 35 hr postpartum.

DISCUSSION

We have exploited the intracellular estrogen receptor characteristic of target tissues for steroid hormones as specific binding reagent for unconjugated estrogen assay in male and female blood plasma (13-16). During human pregnancy, there are markedly elevated concentrations of unconjugated estrogens present which made it possible for us to simplify the assay procedure by elimination of the extraction and chromatographic steps. This reduced the time required for the assay to 3 hr and increased the number of samples, which could be processed enormously, while reducing costs substantially. We were able to show that neither the numerical value nor the precision of the assay was affected by the simplification, also ensuring that we were not measuring estrogen conjugates. Since we could use up to 0.02 ml of plasma, the lower limit of sensitivity of the assay was 1 ng/ml. In the last trimester of pregnancy the vast bulk of determinations were over 8 ng/ml (Fig. 1) and the coefficient of variation of replicates was <10% at this concentration or above.

We found that the mean plasma E₂ concentration rose at a fairly constant rate during pregnancy until the 35th wk when the rate of increment decreased. Values at term tended to become more variable ranging from 5.5 to 50 ng/ml (in one instance). These results confirmed previous reports (20-22) using entirely different assay methods; the validity of our procedure was hence indirectly supported. A declining rate of increase in late pregnancy has been shown to be characteristic of the other principal placental hormones progesterone (23, 24) and chorionic somatomammotropin (8, 25).

Of note is the fact that in studies of normal pregnancy, from the 32nd wk no E₂ level below 5.5 ng/ml was found (Fig. 1) and no significant decline occurred during the course of gestation (Fig. 2). By contrast, in each abnormal pregnancy followed serially (Fig. 3), except in Rh isoimmunization disease, falling E₂ levels and abnormally low concentrations were found, suggesting that particularly in cases of placental insufficiency, following the plasma E₂ may be of clinical assistance. Unfortunately we had no diabetic pregnancies.

The high E₂ concentrations persisting after evidence of fetal demise in Rh isoimmunization disease (Fig. 3) are consistent with values obtained for plasma (7), and urinary estriol (25-28), as well as chorionic somatomammotropin (25), when these assays were used to evaluate fetal well being. It is generally accepted that the placenta is hypertrophied in both Rh isoimmunization disease and many diabetic pregnancies, resulting in persistence of normal hormone values despite clear evidence of prior fetal demise. In cases of placental insufficiency however, the plasma E₂ assay provides as good
evidence of the clinical state as any other hormone measurement.

Estimation of the clinical usefulness of any of the hormonal parameters of fetoplacental well being is difficult because the clinical setting in which the assays are performed is of importance. Under conditions of nearly continuous fetal monitoring, clinical evaluation may be more satisfactory than any test with a time delay. However, as previously emphasized (7, 27–29) a fall of serially obtained urinary or plasma E₂ concentrations strongly suggested imminent fetal distress so that significant fetal salvage might be anticipated by appropriate follow-up procedures in general prenatal-care clinics or particularly in high-risk clinics. Our data indicate that measurement of the plasma E₂ can give similar if not superior information. Therefore it is suggested that this rapid, simple, and inexpensive assay may be of considerable help in identifying and following patients suspected of placental insufficiency.

In study of induced abortion at 14–20 wk gestation, the fall in E₂ concentration after fetal death, which presumably represents the fetal contribution, averaged only 40% (Fig. 4). The persistently high plasma concentrations at 20 hr were probably due to a continued supply of precursors by maternal adrenals and the presence of an intact placenta. Persistence of the plasma progesterone (30) and human chorionic gonadotropin (HCG) (8) under similar conditions attests to the viability of the placenta and supports the quantitative contribution of adrenal sources to E₂ secretion at this time of pregnancy. The patient with suppressed adrenals had a fall of E₂ to 200 pg/ml. The source of this concentration of E₂, a normal luteal phase level, was not clear but presumably ovarian and fetal adrenal sources may contribute.

The half-life of unconjugated E₂ has been reported to be about 20 min based on studies measuring the disappearance rate of intravenous tracers in nonpregnant women (31). We have taken a different approach and measured the disappearance of endogenous hormone from the blood postpartum. Assuming a linear initial disappearance, the mean first half-times of the hormone was found to be 22 min with a range of from 13 to 45 min (Fig. 5). In the four cases, followed for more than 1 hr (Fig. 6), a two compartment model can be shown to characterize the vast bulk of E₂ clearance, with a mean second t₁ of 6–7 hr and a range of from 6 to 8 hr. The E₂ concentration reached follicular phase levels by 14–35 hr, similar to the fall of progesterone (23, 24) at a time when plasma HCG levels remained quite high (32, 33). These data directly support the view that HCG does not stimulate estrogen secretion by the ovary of pregnancy. Previous studies (22), though confirmatory, were not sensitive enough to delineate the lower portion of the curve. The physiological significance of these two compartments is not known.

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