

Unconjugated Estrogens in the Perinatal Period

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Extract

High levels of unconjugated estrone ((E₁) 3-hydroxyestra-1,3,5(10)-triene-17-one) and estradiol ((E₂) estra-1,3,5(10)-triene-3,17β-diol) were found at term in maternal venous, umbilical vein, and umbilical artery plasma. For estrone the respective values in nanograms per milliliter ±SD were 12.8 ± 5.9, 25.1 ± 6.5, and 13.2 ± 7.7. For estradiol the respective values were 17.3 ± 9.2, 8.1 ± 4.0, and 5.1 ± 3.2. For estrone, levels for umbilical vein were higher than those in the paired maternal and umbilical artery; estradiol was higher in maternal vein than in the paired umbilical vessels. Absence of the fetal adrenals was associated with low levels of estrone and estradiol for maternal and umbilical vessels, whereas, in anencephaly, only the maternal levels were markedly diminished. An initially rapid, and then a slower decline in both estrogens was found in normal infants during the first 72 hr of life.

Speculation

Despite extensive metabolism of unconjugated estrogen by the fetus, umbilical arterial levels of estrone and estradiol are high. Nevertheless, the neonate shows scant clinical evidence of those high levels. This could be due to incomplete development of estrogen receptors in target tissues, plasma protein binding of estrogen, competition between the biologically most potent estrogen estradiol and the less potent estrone and estriol ((E₃) estra-1,3,5(10)-triene-3,16α,17β-triol), or other factors.

Introduction

The metabolism of estrogens in the fetoplacental unit has been explored extensively at midterm gestation and was the subject of recent reviews [2, 5]. Less attention has been directed to estrogen metabolism at term, due in part to inability to manipulate the experimental subjects at that time, and in part to the large amounts of serum or plasma previously needed for the laborious methods for estrogen quantitation by classic techniques.

The advent of radioimmunoassay for quantitation of circulating estrogens makes it possible to ask new questions regarding estrogen metabolism at term and in the perinatal period.

The questions posed in this investigation were as follows. (1) What are the levels of unconjugated estrone and estradiol to which the term fetus is exposed, and to what extent does he diminish the level of unconjugated estrogens? (2) How do these levels relate to those of the mother?

Methods

Plasma estrogen levels were determined by a modification of the method described by Hotchkiss *et al.* (10) utilizing Sephadex LH-20 chromatography for the isolation of estrone and estradiol. In brief, this chromatographic step consists of application of the residue of diethyl ether extracts of 5-1,000 μl serum to Sephadex

Table I. Estrogens produced by fetoplacental unit tested for cross-reactivity with antibody

Steroids tested	Estradiol equivalent, ng ¹
2Methoxyestrone	0.007
6 α -OH-estradiol	0.015
15 α -OH-estrone	0.007
15 α -OH-estradiol	0.07
16 α -OH-estradiol	0.02
16Keto-estradiol	1.0

¹ Ten nanograms of each steroid were used; equivalent in terms of estradiol.

LH-20 columns (0.5 \times 6.5 cm) previously equilibrated with the eluting solvent mixture (heptane-chloroform-methanol-water, 10/10/1.5/0.06). Chromatography of ¹⁴C-labeled steroids showed that estrone emerged from the column in the 1.3–3.3-ml fraction, whereas estradiol emerged in the 4.3–8.3-ml fraction. The columns, used repeatedly for 6 months, were stored immersed in column solvent in a closed glass container when not in use, and were recalibrated monthly using ¹⁴C-labeled estrogens.

This chromatographic step isolates and removes some of the compounds known to cross-react weakly with this antiserum [10], as cholesterol emerges in the first milliliter of the eluate, and estriol emerges considerably later than estradiol. Additional estrogens produced by the fetoplacental unit were tested for cross-reactivity with the antiserum (Table I). While 16-keto estradiol caused significant displacement of tritiated estradiol from the antiserum, it would not contribute reactive material to the radioimmunoassay of estrone and estradiol inasmuch as it emerges from the column after estradiol.

Triplicate standard curves for estrone and estradiol (5–100 pg) were prepared in disposable glass culture tubes (12 \times 75 mm) in which 2 or 4 ml, respectively, of column solvent had previously been evaporated.

An antiserum to 17 β -estradiol-succinyl-bovine serum albumin raised in ewes was kindly supplied by Drs. Ferin and Vande Wiele [7].

The radioimmunoassay was performed by the sequential addition of 100 μ l estrogen antiserum (diluted 1/135,000 for the estrone assay and 1/180,000 for the estradiol assay) followed 30 min later by the addition of 100 μ l of a tritiated estrone solution (2, 4, 6, 7-³H-estrone, spec act 95 Ci/mM [26], ca. 10,000 cpm in 0.01 M phosphate buffered saline containing 0.1% gelatin). After overnight incubation of the samples at 4°, separation of the antibody-bound and free steroid was performed as previously described (10). The antibody-

bound steroid was decanted into scintillation vials to which 10 ml toluene-based scintillation medium (7 g 2,5-diphenyloxazole [27] and 0.3 g *p*-bis(*O*-methylstyryl)benzene [26] per liter toluene) were added.

The capped samples were shaken for 1 hr to ensure extraction of the radioactive steroid from the aqueous into the organic phase, and counted in a Packard model 3320 liquid scintillation spectrometer [27] with a tritium counting efficiency of 50–55%.

Blanks

Blank values determined by the extraction, chromatography, and radioimmunoassay of 20–1,000- μ l aliquots of water ranged between 0.7 and 3.6 pg for estrone, and 1.6 and 6.0 pg for estradiol.

Duplicates

All sera were assayed in duplicate, and all samples from an individual mother-infant pair were performed within the same assay. The intra-assay coefficient of variation for 36 pg estrone was 6.3%; for 38 pg estradiol the figure was 6.6% ($n = 10$). Inter-assay coefficients of variation for 61 pg estrone were 6.6%, and for 26 pg estradiol were 9.4%, respectively ($n = 9$).

Recoveries

In each assay, duplicate 50-pg amounts of both estrone and estradiol added to 20–1,000- μ l aliquots of water were carried through the extraction, chromatography, and radioimmunoassay steps to determine procedural losses. The average recovery of estrone was 80%, and of estradiol was 83%. The mean difference in percentage of recovery between duplicates within a given assay was 6% for both estrogens with a range of 1–11% ($n = 10$).

Calculations

The standard curves for both estrone and estradiol were linearized using the logit transformation described by Rodbard (21). The amount of material in the sample was read from the standard curve, the appropriate blank value subtracted, and the result corrected for procedural losses as determined in duplicate for each assay. The final data are expressed as picograms or nanograms of steroid per milliliter of serum.

Experimental Subjects

In the studies performed at delivery, maternal venous plasma was obtained within 5 min before birth from the antecubital vein, and umbilical artery and vein

Table II. Unconjugated estrone and estradiol in paired maternal and umbilical vessel plasma¹

Normal subjects				Estrone, ng/ml			Estradiol, ng/ml		
Initials	Sex	Gestational age, mo	Birth weight, Kg	Maternal vein	Umbilical		Maternal vein	Umbilical	
					Vein	Artery		Vein	Artery
CH	M	38.3	2.9	4.3	23.7	4.7	7.4	5.9	1.9
DA	F	41.6	3.6	7.5	26.3	7.6	9.2	6.7	2.0
DI	F	40.0	3.2	16.4	28.4	<u>26.7</u>	14.7	4.8	7.9
MO	F	42.1	3.7	3.6	<u>15.5</u>	5.4	15.4	2.3	1.8
RA	F	41.3	3.5	16.9	<u>16.0</u>	4.9	10.9	8.4	2.5
RO	F	37.7	2.7	18.5	<u>38.7</u>	<u>15.5</u>	6.9	13.1	4.4
RU	F	40.3	3.6	11.8	<u>26.1</u>	24.2	<u>31.9</u>	5.5	9.4
SA	F	37.1	3.4	<u>20.5</u>	27.1	13.8	<u>30.6</u>	9.9	4.6
TE	F	41.4	3.9	<u>9.7</u>	28.9	19.1	28.6	<u>16.7</u>	4.9
WI	M	40.0	3.6	18.5	20.1	9.9	17.5	<u>7.4</u>	<u>11.3</u>
\bar{X}				12.77	25.08	13.18	17.31	8.07	5.07
SEM				1.97	2.16	2.56	3.06	1.34	1.07
SD				5.91	6.47	7.67	9.17	4.01	3.21
AR ²	F	45	2.9	1.9	20.8	8.9	3.0	6.2	3.2
TH ³	M	39.3	3.3	2.9	6.6	3.2	5.5	3.3	1.7

¹ The highest and lowest values in each column are underlined.

² Anencephaly.

³ Absent adrenals.

plasma from a section of umbilical cord clamped above and below the site to be sampled. Cord clamping occurred 1–2 min after the infants' heel left the perineum. All infants had uncomplicated vaginal deliveries. Specimens were obtained from neonates by heel prick and collected in capillary tubes. Informed consent was obtained from each parent before initiation of each study.

The anencephalic at autopsy had a well developed pituitary, and the adrenal glands weighed 1 g (combined weight).

The male infant with presumed absence of adrenals had three previous brothers who died between 1–3 days of age. Of two who were autopsied, a careful search was made for adrenal tissue and none was found. Our patient had poor circulation at birth, with circulatory collapse at age 2 hr, which responded to glucocorticoid therapy. Cord and amniotic fluid cortisol 1.3 and 1.4 $\mu\text{g}/100$ ml, respectively, and corticosterone sulfate levels of 0.1 and 0.4 $\mu\text{g}/100$ ml, respectively, kindly determined by Drs. Claude Giroud and George Klein (14), were low. The normal mean values for cortisol are 8.6 and 1.8 $\mu\text{g}/100$ ml in cord blood and amniotic fluid, respectively; for corticosterone sulfate those values are 4.4 and 4.8 $\mu\text{g}/100$ ml. Further details of this patient are in press (19).

Results

Studies at term (Table II) indicated that, for unconjugated estrone, umbilical vein levels were always greater than those of the corresponding umbilical artery. In 9 of 10 cases, the umbilical vein levels exceeded those of the maternal vein. The mean value for umbilical vein E_1 was approximately double the value for maternal vein and umbilical artery. Paired comparisons showed umbilical vein to exceed maternal vein ($P < 0.001$) or umbilical artery ($P < 0.0005$).

However, for estradiol, maternal venous levels exceeded those of the corresponding umbilical vein in nine instances. In 7 of 10 subjects, the umbilical vein estradiol exceeded the umbilical artery value. The mean value for maternal unconjugated E_2 was double that found in umbilical vein, the latter being 1.6 times greater than that in the umbilical artery. Paired comparisons showed maternal vein to exceed umbilical vein ($P < 0.025$) and umbilical artery ($P < 0.005$).

Maternal venous levels of estrone and estradiol in one subject bearing an anencephalic infant (AR) and in one subject bearing an infant with presumed adrenal absence were lower than the lowest value observed in the normal maternal group.

In contrast, however, umbilical venous and arterial

Table III. Plasma levels of estrone and estradiol during first 72 hr of life

	Steroid levels at various ages, pg/ml				
	2 hr	12 hr	24 hr	48 hr	72 hr
Estrone					
Mean	1,100	280	150	75	50
SEM	200	65	25	15	10
Number	4	4	9	7	6
Estradiol					
Mean	370	85	70	40	30
SEM	20	20	20	10	10
Number	3	4	10	8	7

levels of estrone and estradiol appeared to be normal in the anencephalic infant (*AR*), whereas these steroid levels were notably lower in the infant with presumed adrenal absence (*TH*). The lowest values observed for the group of normal infant umbilical vessel samples were higher than those observed for *infant TH*.

Studies in Infants between Birth and Age 3 Days

Disappearance Rate of E_1 and E_2 (See also Table III)

Initially there was a very rapid decline in the levels of unconjugated E_1 and E_2 , followed by a more gradual fall. Considering only the portion of the slopes between 24 and 72 hr of age, the times required for a 50% decrease in the values for E_1 and E_2 were approximately 30 and 38 hr, respectively.

Discussion

Diczfalusy [5] and others have shown that the midterm fetus makes an important contribution to the increased production of estrogens during pregnancy by providing precursors, especially 3β -hydroxyandrost-5-en-17-one sulfate (dehydroepiandrosterone sulfate (DHAS)) and 16α -hydroxyandrost-5-en-17-one (16α -OH-DHA [28]) for placental aromatization and conversion to E_1 , E_2 and E_3 [2, 5]. The fetus has an extensive capacity to conjugate estrogen, produced by the placenta, with sulfate and glucuronic acid. Estrogen hydroxylation reactions also take place in the fetus, and 16α -, 16β -, 6α -, and 15α -hydroxy as well as 2-methoxy estrogens have been isolated. This type of study has not been possible at term. It has been hypothesized that the extensive metabolism of the three principal estrogens could serve to protect the midterm fetus from high unconjugated levels of those steroids.

The plasma concentrations of E_1 , E_2 , and E_3 have

been monitored in the mother throughout gestation using radioligand and radioimmunoassay methods [29]. Declining levels of E_2 [25] reflect diminishing supply of fetal steroid precursors and provide information on fetal distress and demise. Because of the limitations of previous methodologies, information is unavailable on actual circulating levels of unconjugated or conjugated E_1 , E_2 , or E_3 in the normal or abnormal fetus during gestation and prior to term.

Umbilical Vessel Levels of Estrone and Estradiol: Normals

Two groups have reported the levels of total estrogens (*i.e.*, conjugated plus unconjugated) in the umbilical artery and vein, and found that umbilical venous levels, coming from the placenta, were not significantly different from those in the umbilical artery coming from the fetus [17, 22]. Klausner and Ryan [13] compared unconjugated and conjugated E_3 in umbilical vein and artery in five babies, and detected no consistent arteriovenous difference in paired specimens. However, Anderson and colleagues found a rapid drop in levels of unconjugated E_1 and E_2 when comparing cord plasma with specimens drawn from the neonate within 1 to 3 min after parturition [1]. The latter findings are consistent with extensive metabolism of unconjugated estrogen by the neonate.

Our new data show that the placenta supplies the fetus (through the umbilical vein) with very high levels of unconjugated estrone and estradiol, the former being of the order of 200 times and the latter 100 times higher than the values in nonpregnant adult females. Despite the extensive metabolism of those estrogens known to occur in the midterm fetus [5], the levels are diminished only by approximately half in the blood returning from the fetus (*i.e.*, umbilical arteries). The method of disposal of unconjugated estrogen presumably involves the conjugating and hydroxylating mechanisms referred to above. That the fetus shows only slight evidence of feminization; *i.e.*, palpable breast tissue, occasional "withdrawal bleeding" during the first few postnatal days, and estrogen effect on vaginal or urethral cytology [23], is surprising. Smaller amounts of circulating estrogen are capable of producing more marked physical evidence of sexual precocity in infants and children. With idiopathic sexual precocity we find levels of E_1 and E_2 which are well within the range for nonpregnant adult females.

It is conceivable that a decreased number of estrogen receptors *in utero* could relate to the lesser re-

sponse of the fetus and neonate to such high levels of unconjugated estrogen. Evidence in support of this concept for uterus, pituitary, and hypothalamus has been obtained in rats. The concentration of uterine estradiol binding sites increases fourfold between *day 1* and *10* and then remains relatively constant through *day 22* [4]. A concentration gradient between pituitary and plasma is observed after *day 10* in rats [11]. In a study starting at *day 5*, a concentration of labeled estradiol in median eminence and hypothalamus calculated as ratio to cerebral cortex is not observed until *day 20-30* [20].

Another possibility is that a binding protein circulates in the fetus and neonate which prevents estrogen from reaching receptor sites. A binding protein with high affinity and high capacity for estradiol is present in rats at *day 5*. The concentration of this protein decreases during maturation in that species (18). No human data are available.

An additional possibility is that competition by other steroids diminishes estradiol binding at tissue sites. Large amounts of progesterone, testosterone, and cortisol do not compete [6]. The estrogen conjugates estradiol-3-sulfate or -17-sulfate do not compete more effectively than estradiol [8]. However, both estrone and estriol reduce the accumulation of $^3\text{H} - \text{E}_2$ in anterior pituitary, uterus, vagina, and hypothalamus (6).

Irrespective of the mechanism involved, it is of considerable interest that no latent deleterious psychologic or physiologic effects result from exposure of the fetal central nervous system to those levels of estrogen, inasmuch as alterations of those functions in the adult are produced by exogenous estrogen administered to immature animals [9, 16]. Significant uptake of tritiated estradiol by the female rat hypothalamus has been demonstrated as early as the 20th day of gestation (15).

Maternal Levels of Estrone and Estradiol: Normals

Our data for maternal unconjugated E_1 and E_2 are similar to those reported by other workers for mothers at term [24]. We suggest that the higher values of E_2 than E_1 could be due to the high levels of sex steroid binding globulin in the mother [3]. This globulin has a greater affinity for E_2 than E_1 .

Maternal and Umbilical Estrogen Levels in Anencephaly and Presumed Absence of Adrenals

In the anencephalic baby, a pituitary gland can nearly always be found, although little or no hypothalamic

tissue is present. Therefore, these babies are deprived of hypothalamic releasing factors including corticotropin releasing factor. Despite this, we have shown that cortisol is produced in anencephaly, although at a subnormal rate [12]. Anatomically, the adrenal glands are small and lack the fetal cortex. The latter is believed to be the source of the estrogen precursors produced by the fetal adrenal, principally DHA and DHAS. Therefore, the levels of urinary estriol are low in mothers carrying anencephalic fetuses [2]. Our new data show that unconjugated E_1 and E_2 were also low in a mother carrying an anencephalic fetus, whereas umbilical venous and arterial steroid levels were not outside the normal range.

In the baby with presumably absent adrenals, the maternal and umbilical vessel levels of both estrogens were below the normal ranges. It is suggested that the complete lack of production of estrogen precursors by this fetus had a greater effect on those levels than that produced by understimulation of the adrenals as in anencephaly. It would be of interest to explore the effects of overstimulation of the fetal adrenals, as in congenital virilizing adrenal hyperplasia (adrenogenital syndrome).

Estrone and Estradiol during First 72 Hr of Age

The initially very high levels of estrogens in the umbilical vessels afforded a chance to follow their disappearance over the first few days of life in neonates. Considering only the portion of the slopes between 24 and 72 hr of age, the time required for a 50% decrease in the values for E_1 and E_2 were 30 and 38 hr, respectively. Others, studying mothers in the immediate postpartum period at comparable times have estimated a 50% disappearance rate of E_2 as 6 hr [25]. Thus, after the initial rapid decline in E_2 in the mother and neonate, and considering only the latter portion of the curve, the data are consistent with the concept that the rate of disposal of the remainder of the estradiol is slower in the neonate than in the mother.

Summary

The utilization of Sephadex-LH 20 chromatography and radioimmunoassay permitted for the first time a comparison of unconjugated estrone and estradiol in paired specimens between the mother and her infant's umbilical artery and vein. The highest levels of estrone were found in the umbilical vein, whereas estradiol was highest in maternal venous blood. A normal mother and her male infant with familial absence of

the adrenals were found to have low levels of both estrogens. Anencephaly of the fetus was associated with marked diminution of only the maternal levels of estrone and estradiol. In the normal infants, the levels of both estrogens dropped rapidly within the first few hours of life followed by a more gradual decline.

References and Notes

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