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Blood Estriol Conjugation During Human Pregnancy*

JOSEPH C. TOUCHSTONE, JOHN W. GREENE, JR., ROBERT C. MCELROY, AND TARAS MURAWEC

From the Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia

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Results of estriol determinations and methods used with blood plasma after enzymatic hydrolysis with β-glucuronidase and phenolsulfatase indicated that a large proportion of estriol in the plasma of pregnant women was present as a sulfoglucosiduronate diconjugate (4.5 μg, 44%). The sulfate (3.1 μg, 25%) and a glucosiduronate (3.8 μg/100 ml, 31%) were also present. Cord blood plasma contains estriol mainly as the sulfate (69.0 μg/100 ml, 70%), with smaller amounts as the glucosiduronate (22.0 μg/100 ml, 24%). "Free" ether-extractable estriol averaged 3.7 μg/100 ml in the maternal blood plasma, and 12.2 μg/100 ml in the cord blood plasma. The red blood cells contained no estriol measurable by the present method.

Evidence for the presence of free estriol in plasma of the human maternal peripheral circulation and of umbilical cord blood has been reported in a previous communication from this laboratory (Touchstone and Greene, 1960). The present paper describes methods used and results obtained in the determination of free and conjugated estriol in blood. Estriol is present in the cord blood in the free form and as conjugates of both sulfuric and glucuronic acids. The sulfate conjugate accounted for 77% of the total estriol. The maternal peripheral blood contains free estriol, estriol sulfate, and estriol glucosiduronate. In addition, maternal peripheral blood contains a diconjugated estriol (a 3-sulfate-16- and/or 17-glucosiduronate), which accounted for 44% of the conjugates.

EXPERIMENTAL PROCEDURE

Extraction of Free Estriol.—Cord blood was obtained by milking the umbilical cord at the time of detachment from the fetus. Maternal blood was procured from the antecubital vein during labor or during the last trimester of pregnancy. In order to keep results within the limits of accuracy of the method, aliquots of 10 ml or more of cord blood were extracted in each experiment while 50 ml or more of maternal blood plasma were used.

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The heparinized blood was centrifuged within 15 minutes after withdrawal, and plasma was separated. The plasma was extracted three times with equal volumes of ether (the plasma, after separation, was kept for hydrolysis studies) over periods of 10 minutes each, with vigorous shaking. The combined ether extracts were washed twice with $^1/_{10}$ volumes of 4% bicarbonate, then extracted three times with $^1/_6$ volumes of 1 N sodium hydroxide, and the ether was discarded. The alkali phase was neutralized with 5 N HCl to *p*H 7.0 and extracted three times with equal volumes of ether. The ether was washed once with $^1/_{10}$ volume of 4% $NaHCO_3$ and twice with water, and was evaporated to dryness. The residue so obtained was subjected to column chromatography for separation of free estriol.

Treatment of the Red Blood Cells.—The cells after separation of plasma were washed twice with isotonic saline using a volume equivalent to the original volume of the plasma. Water was then added to hemolyze the cells. The solution so obtained was precipitated with acetone in the same manner as was the plasma, as described later, and estriol extraction was carried out as described previously.

Hydrolysis of Conjugates.—*In acid hydrolysis*, the residual ether present in the plasma extracted was evaporated *in vacuo* to prevent foaming during hydrolysis. The plasma was refluxed for one hour after addition of concentrated HCl to a concentration of 15% by volume. The precipitate was separated by centri-

TABLE I
RECOVERY EXPERIMENTS
Ten μg estriol added/100 ml plasma (average of 6 experiments).

	Estriol at Start	Loss	Recovery	% Recovery
1. Plasma extracted 3 times with equal volumes of ether, and washing	10	0.3	9.7	97 (92–99)
2. Wash ether 3 times with 1 N NaOH (amount in ether)	9.7	0.5 (loss to ether)	9.2	94 (91–97)
3. Adjust to *p*H 7, extract 3 times with ether, and washing	9.2	0.3	8.0	96 (95–98)
4. Transfer to column and development	8.9	0.9	8.0	90 (86–92)

fugation. The supernatant was extracted as described above. Identical estriol values were obtained when deproteinized plasma was used.

In enzymatic hydrolysis, the plasma proteins were precipitated by addition of 4 volumes of acetone. The precipitate was removed by centrifugation and washed with acetone. The combined acetone was removed *in vacuo* in a warm water bath. The aqueous residue was washed once with one-half volume of heptane-benzene 1:1 for removal of lipids. No estrogen was found in the organic phase. This was backwashed once with a one-third volume of water. In glucuronidase hydrolysis the supernatant was adjusted to *p*H 6.8 with maleate buffer. After addition of 1000 units of glucuronidase (Sigma) per ml of solution, the incubation was carried out at 37° for 18–24 hours. In sulfatase treatment, the aqueous residue was adjusted to *p*H 6.0 with maleic acid buffer and incubated at 50° for 24 hours with 10 mg of Mylase P (Nutritional Biochemicals Corp.) per ml of solution. For each 100 ml of deproteinized plasma, 5 ml of 0.2 M monosodium maleate and 5 ml of 0.2 M sodium hydroxide were added; then the *p*H was adjusted to 6.0 or 6.8 with a *p*H meter. Following hydrolysis, extraction with ether and separation of phenols were carried out as for free estriol.

Column Chromatography.—The method for chromatography on alumina described by Eberlein *et al.* (1958) was modified as follows: 1 g of alumina was used for chromatography in 5-ml syringes fitted with 22-gauge needles. The columns were developed successively with: (1) 10 ml of heptane, (2) 10 ml of benzene, (3) 10 ml of 2% methanol in benzene, (4) 10 ml of 4% methanol in benzene, (5) 15 ml of 30% methanol in benzene. Fraction 5 contained estriol and was evaporated *in vacuo* at a temperature not over 70°. A vacuum oven is convenient when a number of determinations are being done.

Colorimetry with Bachman Reagent.—Phosphoric acid containing 2% sodium *p*-phenol sulfonate for colorimetry of estriol was first described by Bachman (1939). To increase the sensitivity of the method, 2.0-ml cuvettes were used. After evaporation of solvents from the column fractions, 2.0 ml of reagent was added to each flask and heated at 150° for 10 minutes. Absorbance from 400 to 600 mμ was determined on a Beckman DU spectrophotometer. The reagent was stable for several weeks and duplication of results from week to week was excellent. The use of this reagent is limited to concentrations of estriol from 0.5 to 5.0 μg/ml of reagent as shown in Figure 1. Above this concentration, Beer's law did not hold.

Recovery Experiments.—Table I shows the recovery of estriol in each step in the four-step procedure used to prepare extracts for colorimetry. The greatest loss in the method occurred in the chromatography on alumina. Recovery for each other step in the procedure is given in the table. Over-all recovery was 80%. Pooled pregnancy plasma samples were made up to a concentration of 10 μg per 100 ml of plasma.

Specificity of the Method.—The specificity of the method depends upon the extraction and purification procedures employed, the Bachman color reaction and on the enzymes employed.

The formation of a violet-pink color with an absorption maximum at 545 mμ and an extinction coefficient ($E^{1\%}_{1mm}$) of approximately 600 at 545 mμ is a specific property of estriol. No other phenolic steroid than 16-epiestriol has been found to give this chromogen. Under conditions of development of the alumina column 16-epiestriol is largely removed. Studies with paper chromatography on the estriol fraction from the column have indicated that no 16-epiestriol is present. Gas chromatography of the estriol fraction from cord blood after Mylase-P treatment and treatment on the alumina column showed only estriol (Fig. 2).

The spectral characteristics of the Bachman color produced by pure estriol and by the estriol isolated from blood are shown in Figure 3. The maximum at 545 mμ was shown by the estriol from the free and conjugated fractions of both cord and maternal blood.

Precision of Method.—The method of Snedecor (1952) was used to give an estimate of the precision of the determinations. The standard deviation of results of duplicate determinations from their means was calculated as follows.

$$SD = \sqrt{\frac{d^2}{2N}}$$

where d is the difference between two results in a duplicate determination, and N is the number of duplicate determinations performed. The standard deviations in nineteen duplicate determinations of estriol in cord blood plasma in concentrations ranging from 30–83 μg/100 ml of plasma was 5.5 μg/100 ml. For maternal blood plasma the standard deviation in concentrations of 3–13 μg/100 ml was 0.5 μg/100 ml in eight duplicate determinations.

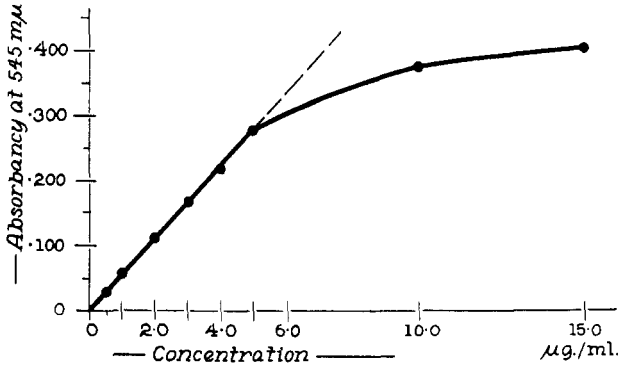

FIG. 1.—Estriol calibration curve.

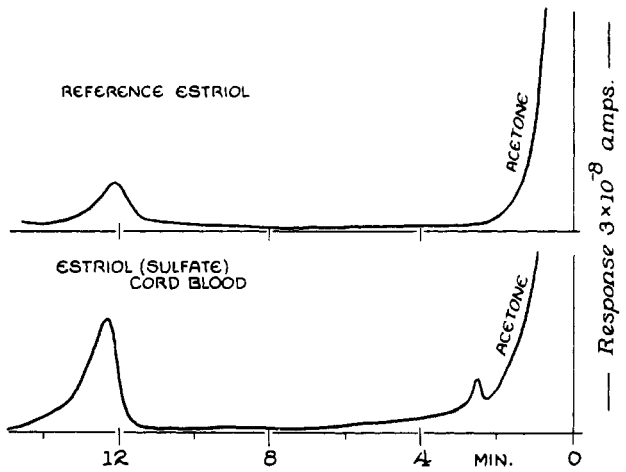

FIG. 2.—Gas chromatography of estriol. Conditions: glass coil column 6 ft × 4 mm; 3% QF-1 on gas chrom Z, 100–140 mesh; argon pressure, 23 psi; temperatures: column, 240°, detector, 250°, flash, 270°. Chart speed 0.5 in./min ("Chromalab," Glowall Corp. Glenside, Pennsylvania).

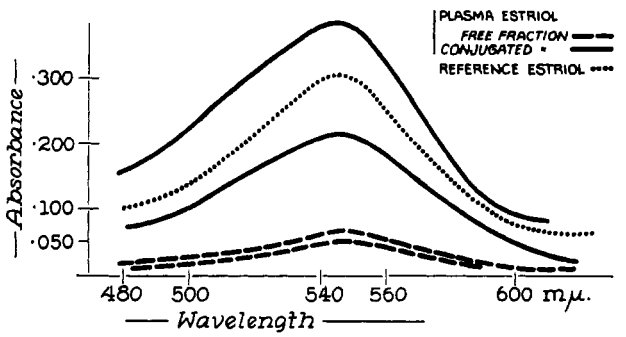

FIG. 3.—Absorption spectra of reference and isolated estriol after Bachman reaction.

Identity of Estriol.—The absorption spectra of the estriol (liberated after either enzyme hydrolysis) in alcohol, in sulfuric acid, in the Bachman reaction, and in the Kober reaction were identical with those presented by reference estriol under equivalent conditions. The mobilities in the paper chromatographic systems methylene chloride–formamide and isopropylether–ethylene glycol were identical with those shown by reference estriol. The reaction with ferric chloride–potassium ferricyanide and sulfuric acid performed on the zones on the paper chromatograms was characteristic. The fluorescence and excitation spectra in sulfuric acid were identical with those of reference estriol. The isolated estriol gave a methylether which was eluted in the same fraction from an alumina column as authentic estriol methylether. The acetate had a mobility in toluene–propylene glycol paper chromatograms like that of reference estriol acetate. Isolated estriol showed a retention time like that of reference estriol on gas chromatography (Fig. 2).

RESULTS

The free, ether-extractable estriol present in fifteen samples of whole plasma averaged 3.7 μg/100 ml in maternal and 12.2 μg/100 ml in cord blood plasma. The amount of estriol found after acid hydrolysis of maternal plasma averaged 13.7 μg while the cord plasma had an average of 72.9 μg/100 ml. Values as high as 164.1 μg/100 ml of cord blood plasma have been found.

Figure 3 shows the absorption spectrum of the color formed in the Bachman procedure by estriol from the free and acid-hydrolyzed fractions of both cord and maternal plasma. A yellow background color from maternal plasma sometimes interfered with absorbancy measurements at 545 mμ. Preliminary washing of the plasma with heptane–benzene 1:1, as described, removed much of this background.

There was little if any estriol, free or conjugated, detected in the red cells of either the cord or maternal blood by the present method. Based on results found this is less than 0.5 μg/100 ml of the red cell volume.

Enzymatic hydrolysis of whole plasma gave only 70% of the estriol obtainable by acid hydrolysis. Prior to enzymic hydrolysis, plasma proteins were precipitated by addition of four volumes of acetone. There was no difference between estriol values obtained by acid hydrolysis before and after deproteinization. No estriol was found after acid hydrolysis of the precipitated protein. Maleic acid buffer was used in incubations with both enzymes since phosphate has been shown to cause inhibition of phenolsulfatase (Dodgson and Spencer, 1953; Levitz *et al.*, 1961). The present work confirmed this. Table II gives the values for estriol found after hydrolysis of cord blood by β-glucuronidase and Mylase P. The estriol obtained from its sulfate which averaged 69 μg/100 ml of plasma (range 48–112) was the predominant conjugate. The estriol obtained after glucuronidase hydrolysis averaged 22 μg per 100 ml of plasma (range 15–30).

The estriol obtained from its glucosiduronate averaged 3.8 μg and the sulfate 3.1 μg/100 ml in the maternal plasma. However, results shown in Table III indicated that there may be a conjugate other than the sulfate or the glucosiduronate. Values for estriol after glucuronidase hydrolysis following cleavage with Mylase P were higher than when glucuronidase digestion was carried out first. Mylase P hydrolysis results were higher after primary treatment with glucuronidase. Hydrolysis with acid following the two sequential enzyme digestions liberated no more estriol, as shown in Table III.

The results were not due to incomplete hydrolysis in the first incubation, since a second incubation with the same enzyme released no further estriol in either cord or maternal plasma, as shown in Table IV. A single hydrolysis with enzyme gave complete cleavage. If only a sulfate and glucosiduronate were present, acid or glucuronidase hydrolysis after sulfatase treatment should result in an estriol value equivalent to that obtained by glucuronidase digestion initially. Conversely, acid or sulfatase hydrolysis following treatment with glucuronidase should give an estriol value the same as sulfatase hydrolysis performed first. Since acid hydrolysis after sequential incubation with both enzymes showed no further increment of estriol it appears that the sulfate and glucosiduronate are the

TABLE II
ESTRIOL CONJUGATION IN CORD BLOOD PLASMA
(μg/100 ml plasma)

No. of Subjects	Sulfate[a]	HCl after M[b]	Glucosiduronate[c]	HCl after G[d]
4	86.9 ± 10.7[e]	23.2 ± 3.5	22.0 ± 3.3	77.5 ± 7.8
12	69.0 ± 5.1	—	22.1 ± 1.4	—

[a] Estriol liberated by Mylase P. [b] Acid hydrolysis following the Mylase-P treatment. [c] Estriol liberated by β-glucuronidase. [d] Acid hydrolysis following the β-glucuronidase treatment. [e] Mean ± standard error.

TABLE III
ESTRIOL CONJUGATION IN MATERNAL BLOOD PLASMA
(μg/100 ml plasma[a])

	N[b]	Sulfate	Glucosid-uronate	HCl	Total
Procedure A[c]	3	3.8 ± 0.2[d]	14.8 ± 4.0	0	18.4 ± 1.2
Procedure B	2	10.8 ± 1.6	5.2 ± 1.2	0	16.0 ± 2.8
Procedure C					
(1)	11	3.1 ± 0.5	8.4 ± 1.9[e]	0	11.5 ± 1.7
(2)	11	7.7 ± 1.1	3.8 ± 0.4[e]	0	11.5 ± 1.6

[a] Measured as free estriol liberated by hydrolytic procedures. [b] Number of samples. [c] (A) Mylase P followed by β-glucuronidase; (B) β-glucuronidase followed by Mylase P; (C) 1 and 2 on same blood sample. [d] Mean ± standard error. [e] Double conjugate calculated to be 4.5 ± 0.7 μg/100 ml by difference between (1) and (2).

TABLE IV
EFFICIENCY OF HYDROLYTIC PROCEDURES
(μg estriol/100 ml)

Blood Sample[a]	Mylase	Mylase II[b]	HCl[c]	Glucu-ronidase	Glucu-ronidase II[b]	HCl[e]
(1) Maternal	4.0	0	10.0	6.0	0	10.0
(2) Maternal	2.5	0	7.6	4.2	0	6.6
(3) Maternal	3.0	0	6.2	5.1	0	5.5
(4) Cord	65.0	0	—	15[d]	0	—
(5) Cord	65.0	0	—	15[d]	0	—

[a] Pooled blood samples divided into two portions for each enzyme experiment. [b] Reincubated with additional enzyme. [c] Acid hydrolysis after two incubations with Mylase P. [d] Glucuronidase hydrolysis carried out after Mylase P hydrolysis. [e] Acid hydrolysis after two incubations with β-glucuronidase.

major forms of conjugation, and therefore these results suggest that some of the estriol is conjugated with both sulfuric and glucuronic acid, presuming there are no other enzymes present in the preparations. Phosphatase is present in the β-glucuronidase and probably other enzymes, but no other estriol conjugates have been demonstrated.

Free estriol represented 17% of the total estriol found in the maternal plasma. The conjugated fraction averaged 83% of the total, of which 27% was glucosiduronate, 20% was sulfate, and 44% appeared to be the double conjugate which averaged 4.5 μg/100 ml of plasma (Table III).

DISCUSSION

The sulfate was the major conjugate of estriol in cord blood. The glucosiduronate was also present. Although both estriol sulfate and estriol glucosiduronate were present in maternal blood, greater concentrations of estriol were present as a sulfoglucosiduronate diconjugate.

Purdy *et al.* (1961) found estrone sulfate in blood after administration of labeled estradiol to humans. Menini and Diczfalusy (1961) isolated estriol-3-sulfate from human meconium; Kinsella *et al.* (1955) isolated estriol from human meconium after hydrolysis with bacterial β-glucuronidase; and Levitz *et al.* (1961) presented evidence that sulfurylation was a major conjugating mechanism in the human fetus. Since this work was completed, Troen *et al.* (1961) reported the isolation of estriol-3-sulfate from cord blood. Evidence for a sulfoglucosiduronate in small amounts was also presented.

In addition to the conjugates, free estriol was found in both cord and maternal blood. The results as previously reported (Touchstone and Greene, 1960) are in agreement with those of Diczfalusy and Magnusson (1958). Oertel and Eik-Nes (1959) and Roy and Brown (1960) have also described results of measurements of estrogen in blood.

The results obtained with red cells are essentially in agreement with those of Roy and Brown (1960), who showed that identical results were obtained whether the plasma or whole blood were extracted. In a preliminary report Wiggins and Preedy (1961) found no estrone, estradiol, or estriol associated with the red cells of maternal blood in the human.

The postulated sulfoglucosiduronate diconjugate may represent an intermediary in the conversion of estriol sulfate from the cord blood to the glucosiduronate in maternal blood for excretion in the urine. Twombly and Levitz (1960) reported radioactive estrone glucosiduronate (in urine) after administration of the sulfate, while Beer and Gallagher (1955) observed estriol glucosiduronate after administration of radioactive estradiol. Straw *et al.* (1955) and Jayle *et al.* (1959) obtained evidence for the presence of a sulfoglucosiduronate conjugate of estriol in human pregnancy urine in small amounts, thus pointing out that this substance may have passed the renal barrier.

Conclusions in the present work are dependent in part on the specificity of the phenolsulfatase which was shown by Cohen and Bates (1949). Present methods do not make it possible to differentiate between a 16- and/or 17-glucosiduronate. Felger and Katzman (1961) have reported studies on the separation of mono- and diglucosiduronates from urine extracts. Marrian (1937) had earlier showed that the glucuronic acid moiety was probably present at the 16 or 17 positions of estriol. However, Beling (1962) indicated the presence of estriol-3-glucosiduronate in urine. Elucidation of the exact nature of the postulated estriol-3-sulfate-16- and/or 17-glucosiduronate will require rigid chemical characterization.

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Unique Effects of DDT and Other Chlorinated Hydrocarbons on the Metabolism of Formate and Proline in the Housefly*

Richard E. Cline and George W. Pearce

From the Technical Development Laboratories, Technology Branch, Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Savannah, Georgia

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Insecticide-treated and control flies were injected with C^{14}-labeled biochemicals, and 3 hours later the soluble radiometabolites were extracted for identification and assay by paper chromatography and radiometric techniques. Of the injected compounds, DDT was found to interfere most with the metabolism of formate, glycine, and proline. Thus after injection of C^{14}-formate, more uric acid and allantoin and less proline were recovered as radiometabolites from flies treated with DDT and related chlorinated hydrocarbon insecticides than from flies untreated or treated with nontoxic analogs. However, insecticides of other types such as pyrethrum, organic phosphates, and phosphonates and a carbamate, interestingly, failed to show a significant effect on formate metabolism, providing additional evidence for a different mode of action.

Although introduced about 20 years ago, DDT[1] still has the largest share in the volume of insecticides used throughout the world, and little has been learned about how this compound and other chlorinated hydrocarbon insecticides produce their toxic effects. This lack of knowledge concerning the mechanism of action greatly hampers efforts to evaluate the toxicity of such agents toward higher animals, as well as studies of the nature of insect resistance.

The presence of DDT as a trace component of fat in normal humans in many countries has become a well-established fact, but not one which is generally viewed with alarm by toxicologists, since experiments involving the exposure of human volunteers to relatively large amounts of DDT have not revealed toxic effects (Hayes *et al.*, 1956, 1958). However, until more knowledge of the mode of action of these agents has been obtained, there will remain some degree of uncertainty regarding the effects of long-term exposure to such materials.

Numerous data show a high degree of correlation between structure and activity in the case of DDT and related compounds (Metcalf, 1955), but conclusive biochemical evidence showing any specific enzyme or metabolic pathway to be the site of action of such compounds has yet to be found (Metcalf, 1955; Perry, 1960a,b). Strangely, *in vivo* studies involving normal body constituents tagged with radiocarbon appear to have been largely neglected in this field, with the exception of a report by Winteringham (1958) that DDT resembled an organophosphate in its effect on C^{14}-acetate metabolism in the housefly. Tracer techniques and paper chromatographic methods applicable to pesticide research have been discussed (Winteringham, 1960).

The present report describes *in vivo* housefly experiments involving thirteen C^{14}-labeled amino acids, purines, and their precursors. DDT and other toxic chlorinated hydrocarbon insecticides, in contrast to nontoxic analogs and other classes of insecticides, were found to be unique in stimulation of radiopurine synthesis and radioproline degradation in flies injected with C^{14}-formate.

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[1] Abbreviations and insecticide names: AIC, aminoimidazole carboxamide; DDA, bis(*p*-chlorophenyl)acetic acid; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DMC, bis(*p*-chlorophenyl)methylcarbinol; *o,p'*-DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane. For abbreviations and names of other insecticides see Kenaga (1960).