Phenolic Steroids in Human Subjects III. Estrogens in Plasma of Pregnant Women.*† (25010)

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Data on concentration of the various estrogens in human plasma during pregnancy are meager. Furthermore, much of the work was carried out with methods lacking sensitivity and specificity for the low concentrations of these steroids in plasma. Aitken and Preedy (1) determined levels of circulating estrogens during the last 4 weeks of pregnancy and found the following values, expressed as μg/100 ml of plasma: estrone, 2.6-10.3; estradiol-17β, 1.2-2.9; estriol, 4.3-17.5. In discussion following the paper (1) Loraine quoted unpublished data of Roy and Brown who found approximately one-half as much estrogen in plasma as Aitken and Preedy. Diczfalusy in discussing the same paper (1) reported concentrations obtained in "so-called retroplacental blood, *i.e.*, on vaginal blood following delivery." His values, expressed as μg/100 g of blood, were as follows: unconjugated-estrone, 1-3, estradiol-17β, 2-6, estriol, 3-13; conjugated-estrone, 1-4, estradiol-17β, 0.1-0.3, estriol, 4-20. In this paper data on estrogen levels in human plasma during the second and third trimesters of pregnancy are presented. Identity of various estrogens has been established by isotopic dilution and by paper chromatography.

Methods and materials. Approximately 30 ml of blood were drawn from a number of pregnant women and mixed with heparin. Plasmas were separated from erythrocytes, pooled and stored frozen (–10°C) until 1-2 liters were accumulated. After thawing, proteins were precipitated by adding ethanol to final concentration of 80%. After standing at 5°C overnight, precipitate settled leaving a clear layer. The latter was drawn off and the residue centrifuged. After washing precipitate with twice its volume of absolute ethanol, combined supernatants were filtered and ethanol was removed *in vacuo* at a temperature less than 40°C. Radioactive estrogens (0.02 μC, 1-9 μg of 16-C^{14}-labelled estrone, estradiol and estriol) were added to 2 of the deproteinized plasmas at this point. (In the other case they were added just before countercurrent distribution.) Upon standing overnight at 5°C, fat rose to the top and solidified. It was lifted out and discarded. Deproteinized plasma was extracted twice with equal volumes of hexane and the hexane discarded. Residual hexane in the aqueous phase was blown off with air. The deproteinized plasma, adjusted to pH 5 with acetate buffer, was incubated with β-glucuronidase (1000 units of Ketodase/ml) for 72 hours at 37°C. After pH had been lowered to 1 with 60% sulfuric acid, continuous extraction with ether was performed for 48 hours. Concentrated hydrochloric acid (0.15 volume) was added to the boiling aqueous solution and the mixture refluxed for 45 minutes. The cooled hydrolyzate was extracted 3 times with 1/4 volume of ether. The ether was evaporated and the residue, combined with that from continuous ether extraction, was dissolved in 100 ml of ethyl acetate, washed twice with 1/5 volume of saturated sodium bicarbonate solution and once with 1/10 volume of water. The ethyl acetate was removed *in vacuo*. The residue was separated by 99 transfer countercurrent distribution in the system 70% methanol/60% carbon tetrachloride-40% chloroform. Analysis of both fluorescence (unpublished) and radioactivity was performed (2). The contents of tubes under each peak were pooled. Infrared spectrophotometry was attempted, but the fractions were too impure. Consequently, each pool was chromatographed on paper in the systems benzene/55% methanol, or 33% benzene-67% Skellysolve C/80% methanol. Each chromatogram was scanned in an Actigraph for localization of radioactive

* This study was supported in part by grant from Nat. Inst. Health, U. S. P. H. S.

† We are indebted to Dr. Mortimer Levitz and Am. Cancer Soc. for estriol-16-C^{14} and to Jean Valvo, Maria Karsay and Lawrence Beecher for technical assistance.

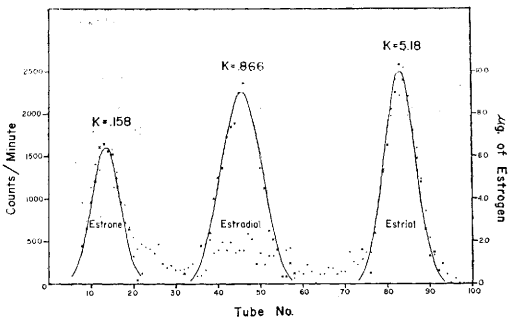

FIG. 1. Countercurrent distribution of an extract of 1.8 liters of plasma from women 28-35 wk pregnant. System: 70% methanol/60% carbon tetrachloride-40% chloroform. × radioactive assay, ● fluorimetric assay. Solid curves are theoretical curves drawn to the partition coefficients (K) given above each curve.

estrogens. One-half of the paper strip was developed colorimetrically with Folin-Ciocalteu reagent(3). Appropriate areas from the other half of strip were eluted, dried, and the residues acetylated with acetic anhydride and pyridine at room temperature overnight. Estrone acetate was rechromatographed and the Zimmermann reaction used to localize the steroid. Estradiol diacetate and estriol triacetate was partially saponified in 0.5 N methanolic potassium hydroxide at room temperature for 30 seconds. The solution was neutralized with acetic acid, diluted with water and extracted with ether. This procedure removes the phenolic acetate group, making the compounds again reactive to the Folin-Ciocalteu reagent. The partially acetylated compounds were chromatographed.

Results. A countercurrent distribution of plasma extract is shown in Fig. 1. In general, there was excellent correlation of radioactivity and fluorescence (except for estradiol in the instance shown), the latter expressed in μg of each estrogen. Analysis of countercurrent distribution data by the method of Baggett and Engel(4) also indicated identity of endogenous hormone and added radioactive steroid. Paper chromatography of the alcohol and its acetate indicated in each case the identity of the radioactive and endogenous steroid. Thus, although we failed to obtain adequate infrared spectra, the identification of the compounds is adequately established.

Two women in each group were taking diethylstilbestrol, but this compound does not fluoresce. In addition, these women contributed less than 2% of the plasma analyzed. None of the women was taking ethinylestradiol, which does fluoresce and which would be difficult to distinguish from estradiol by countercurrent distribution. There were also other fluorogens present. There was a very definite shoulder at tubes 20-25 in each of the specimens studied. In the case of the second trimester plasma extract, there was also a pronounced peak at tube 93. Between the estradiol and estriol areas a varying amount of fluorescence was present in all the extracts examined. It is not known whether these fluorogens are steroidal.

Results of 3 experiments are tabulated in Table I. Concentrations of each of the 3 estrogens were calculated in 2 ways: on the basis of total area of each fluorescent curve and by dilution of added isotopic estrogen. There was excellent agreement of the 2 methods. The amount of estrone and estradiol obtained from plasma collected during the last 4 weeks of pregnancy agrees with the values reported by Aitken and Preedy(1), but our value for estradiol is higher. Our values for concentration of estrone and estradiol during this period are consistent with those of Diczfalusy for whole blood(1), but our estriol value is lower.

It is of special interest to note the relative constancy of estrone and estriol concentrations during the last 2 trimesters of pregnancy, especially in view of the 10- to 100-fold increase in urinary estrogens during this period (5). Estradiol concentration, on the other hand, was quite variable. There was a small amount of estradiol present in the extract from the second trimester plasma, none during the first part of the third trimester (Fig. 1), and a large amount during the last 4 weeks of

TABLE I. Concentration of Estrogens in Human Pregnancy Plasma.

Period of pregnancy, wk	Vol of plasma processed, ml	μg/100 ml of plasma		
		Estrone	Estradiol-17β	Estriol
14-27	2200	5.1	<1.4	4.3
28-35	1800	3.1	< .1	5.1
36-40	1100	9.3	8.2	6.1

pregnancy (Table I). We have no explanation for this.

Summary. By means of countercurrent distribution and paper chromatography, estrone, estradiol and estriol were separated from extracts of one to 2 liters of human pregnancy plasma, quantitated and identified by fluorimetric and isotopic technics. Concentration of estrone and estriol remained more nearly constant (3-9 μg/100 ml of plasma) through second and third trimesters, but the concentration of estradiol varied from 0-8 μg/100 ml.

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Received May 4, 1959. P.S.E.B.M., 1959, v101.

Enhancement of Lethal Action of Endotoxin in Mice by Triiodothyronine. (25011)

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Mice survive lethal doses of endotoxin when pre-treated with cortisone(1). Consideration of interrelationship between thyroid hormone and resistance of mice to endotoxin resulted from observations in dogs in that cortisol production and metabolism were significantly increased by analogs of thyroxin, such as triiodothyronine and triiodothyroacetic acid. In our studies for publication it was found that the magnitude of the rise in cortisol production simulated secretory response of the adrenal to exogenous corticotropin. Others(2) previously reported that following injection of l-thyroxine into guinea pigs urinary output of 17 hydroxycorticosteroid was in direct proportion to dose of thyroxine. Because triiodothyronine increased output of cortisol in the intact animal, it was anticipated that its administration to mice would protect them against lethal action of endotoxin, but on the contrary, triiodothyronine enhanced this lethal action.

Methods. Purified endotoxins were prepared from cultures of *Escherichia coli, Salmonella typhosus* and *Brucella abortus,* as described elsewhere(1). Male ABC mice weighing approximatley 20 g were given endotoxin intraperitoneally in dose of 0.2 to 0.4 mg/20 g body weight. The dose of endotoxin selected for each experiment was that amount which did not produce death in normal mice within 48 hours. A solution of 3:5:3′-l-triiodothyronine was prepared to yield a concentration of 40 μg/0.1 ml.* Groups of 10 mice each were used in a series of experiments. In the first experiment, 20 mice were given 40 μg of triiodothyronine intraperitoneally, and 18 hours later this dose was repeated, a total of 80 μg within 24 hours. Preliminary metabolic studies revealed that within 6 to 12 hours after final injection metabolism was accelerated, reaching a peak within 24 hours. Then, 10 mice served as controls, and the 10 remaining animals received intraperitoneally 0.3 mg/20 g of *E. coli* endotoxin. An additional group of 10 mice received only endotoxin. In a second series of experiments the same procedure was carried out except that 0.4 mg/20 g of endotoxin prepared from *Br. abortus* was used. Similarly, in the third series, 0.2 mg/20 g of endotoxin made from *S. typhosus* #360 was injected.

Results. Results of 3 experiments using 3 different preparations of endotoxin are presented in Table I. Triiodothyronine consistently enhanced the lethal action of endotoxin. Deaths were recorded as having taken place within 48 hours after mice had received endo-

* Supplied through courtesy of Smith, Kline and French Labs., Philadelphia, Pa.