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Perfusion Studies of the Human Placenta. V. Metabolism of [^{3}H]Estrone [^{35}S]Sulfate and [^{14}C]Estradiol-17β at Mid-Pregnancy*

Carmen Alonso and Philip Troen

ABSTRACT: Four placentas from the fifth to sixth month of normal pregnancy were perfused *in vitro;* two with [6,7-^{3}H]estrone [^{35}S]sulfate and two with [^{14}C]estradiol-17β.

Extracts of perfusates and placental tissue were analyzed for free and conjugated estrogens. Approximately 99% of the recovered ^{3}H and ^{14}C radioactivity was in the free form indicating the presence of a phenolsulfatase activity in the placenta. Interconversion of estrone and estradiol was evident from analysis of the free estrogen fraction. A polar metabolite was found not to be estriol but rather [^{3}H]6-hydroxyestradiol-17β and [^{14}C]6-hydroxyestradiol-17β in yields of slightly more than 2% of the recovered radioactivity. This is the first demonstration of 6-hydroxylation of a phenolic steroid by the human placenta.

The finding of preponderantly conjugated estrogens in human cord blood (Troen *et al.*, 1961) in combination with a large amount of free estrogens in placental tissue (Diczfalusy and Lindkvist, 1956) suggested that human placental tissue might be capable of metabolizing estrogen sulfates. [^{3}H]Estrone [^{35}S]sulfate was therefore perfused through placental tissue to determine whether hydrolysis takes place and, if possible, whether metabolism of the estrogen portion of the conjugate preceded hydrolysis. The nature of the unconjugated radioactive estrogens recovered was also determined. Because the pattern of free estrogens recovered from these perfusions was similar to that obtained in separate perfusions with [^{14}C]estradiol-17β, both studies are presented here. These studies utilized placentas obtained at mid-pregnancy, thus allowing comparison with the results previously reported on the metabolism of perfused [^{14}C]estradiol-17β in placentas obtained at term (Troen, 1961).

The major findings to be reported in this paper are the hydrolysis of the estrone sulfate and the formation of free 6-hydroxylated metabolites from both precursors, [^{3}H]estrone [^{35}S]sulfate and [^{14}C]estradiol-17β.

* From the Departments of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts, and Montefiore Hospital and University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. *Received May 10, 1965; revised September 27, 1965.* Supported in part by grants from the U. S. Public Health Service (AM-04290 and AM-08375), the Medical Foundation of Boston, the Guggenheim Memorial Foundation, and the Anathan Research Fund. Preliminary reports of portions of this study have appeared (Troen and Alonso, 1963; Alonso and Troen, 1964).

Experimental Procedure

Four intact placentas were obtained at about the twentieth week of pregnancies interrupted for psychosociologic reasons. There was no evidence of medical abnormality during the pregnancy. The placentas were transported to the laboratory and perfused as previously described (Troen and Gordon, 1958) except that the perfusing fluid volume was reduced to 500 ml and the rate of perfusion was slower, averaging 100 ml/min. [6,7-^{3}H]Estrone [^{35}S]sulfate containing 0.45 μcurie of tritium and 1.1 μcuries of ^{35}S was added to each hourly change of perfusion fluid for perfusion 1, and [^{3}H]-estrone [^{35}S]sulfate with 0.385 μcurie of tritium and 0.95 μcurie of sulfate was added to each hourly change of perfusion fluid for perfusion 2. The recycling perfusions were carried out for 8 hr. The double-labeled estrone sulfate was synthesized and kindly supplied by Dr. M. Levitz of New York University (Levitz, 1963). It was demonstrated to be over 99% radiochemically pure. The ratio of ^{35}S:^{3}H (in counts per minute and corrected for decay) of the starting material was found to be 0.12. Perfusion 3, lasting 40 min, was performed with 0.625 μcurie of [^{14}C]estradiol-17β. Perfusion 4 was conducted for 7 hr with 0.50 μcurie of [^{14}C]estradiol-17β added to each hourly change of perfusing fluid. The [^{14}C]estradiol-17β was demonstrated to be over 99% radiochemically pure. Human chorionic gonadotropin (generously supplied by Ayerst Laboratories) was used in all four placental perfusions in a concentration of 20,000 IU/l. of perfusion fluid.

The perfusates from each placenta were pooled and reduced in volume by evaporation at low pressure. The

TABLE I: Recovery in Placental Tissue and Perfusates of Perfused ^{3}H and ^{14}C Radioactivity Following Perfusions of Human Mid-Pregnancy Placentas with [^{3}H]Estrone [^{35}S]Sulfate (Perfusions 1 and 2) and [^{14}C]Estradiol-17β (Perfusions 3 and 4). Partition of Recovered Radioactivities in Ether-Soluble and Water-Soluble Fractions is Shown.

Perfusion	Perfused (counts/min)	Recovered (counts/min)		% Recovery	Distribution of Recovered Radioactivity (%)	
		Placenta	Perfusate		Ether Soluble	Water Soluble
1	1,029,000	405,800	517,700	90	99.0	1.0
2	880,000	284,700	472,600	86	98.8	1.2
3	344,000	175,600	136,200	91	98.8	1.2
4	1,920,000	534,600	1,044,300	82	98.7	1.3

placentas were minced separately and extracted four times with five volumes of 80% ethanol. The ethanolic extracts of the placental tissue and the perfusate concentrates were kept at −17° until processed. The extraction methods followed were similar to those previously reported (Troen *et al.*, 1961, 1965). After removal of proteins and lipids, the radioactivity was ultimately fractionated into ether-soluble and water-soluble fractions.

Countercurrent Distributions. Countercurrent distributions (CCD)[1] and calculations were performed as previously described in this series (Troen *et al.*, 1965).

Measurement of Radioactivity. Measurement of ^{3}H and ^{35}S radioactivity in the [^{3}H]estrone [^{35}S]sulfate perfusions was performed in a Packard Tricarb liquid scintillation spectrometer Model 314E. Efficiencies were 13% for ^{3}H and 40% for ^{35}S when counting both isotopes simultaneously and 42% for ^{3}H when counted alone. ^{14}C radioactivity was measured with a thin-window Geiger flow detector with an efficiency of 25% as previously described. In a few instances, a Packard Tricarb liquid scintillation spectrometer Model 3314 with an efficiency for ^{14}C of 80% was used. For scintillation counting, the radioactive material was dissolved in 1 ml of ethanol and 14 ml of redistilled toluene containing 0.3 g/l. of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POPOP) and 5.0 g/l. of 2,5-diphenyloxazole (PPO). The simultaneous equation method (Okita *et al.*, 1957) was used to obtain the ratio of ^{3}H:^{35}S in the samples and determinations of quenching were made by adding internal standards to the samples. The stability of the instrument was determined daily. Sufficient counts were accumulated for the standard error to be 2–3%. The ^{35}S counting was corrected for decay to a common reference time.

Determination of Estrogens. Estrogens were analyzed using a modified Brown procedure (Diczfalusy and Magnusson, 1958). A slight further modification was introduced in the partition of estrogens between benzene–petroleum ether (bp 30–60°) and 2% ethanol–water by backwashing the water phase with benzene–petroleum ether to ensure complete separation of the estradiol and the estriol. Ordinarily this procedure recovers an additional 2% of the estradiol from the estriol-containing phase (Brown, 1955). For quantitative determination of carrier estrone, estradiol, and estriol, the Kober reaction was used under the conditions of Nocke (1961); for 6-hydroxyestradiol and its derivatives, the conditions of Breuer *et al.* (1960) were used.

Hydrolysis. Acid hydrolysis was carried out with hydrochloric acid as described by Brown (1955). Enzymatic hydrolysis with phenolsulfatase using Sigma Type III sulfatase was carried out as described in the preceding paper in this series (Troen *et al.*, 1965).

Standards. Reference 6-hydroxyestradiol-17β was obtained by sodium borohydride reduction of 6-keto-3,17β-diacetoxyestradiol (Wintersteiner and Moore, 1959), supplied by Klyne, and subsequent deacetylation of the 6-hydroxy-3,17β-diacetoxyestradiol (Breuer and Knuppen, 1960). The 6-hydroxy compound obtained by reduction with sodium borohydride of the 6-keto compound has been demonstrated to be the α-epimer (Breuer *et al.*, 1961). Another source of 6α-hydroxyestradiol-17β was deacetylation of the 6α-hydroxyestradiol-17β triacetate also supplied by Klyne. The purity of the 6α-hydroxyestradiol-17β used as reference compound was tested by countercurrent distribution in two different systems. Standard estrone sulfate was supplied by Levitz.

Derivative Formation. Methylation and acetylation were carried out as previously described (Troen *et al.*, 1961). Deacetylation was carried out with potassium hydroxide in methanol at room temperature overnight (Breuer and Knuppen, 1960). Oxidation was carried out with chromic oxide in glacial acetic acid at room temperature and in darkness for 2 hr (Lieberman *et al.*, 1953). Reduction was accomplished with sodium borohydride in methanol at room temperature overnight (Wintersteiner and Moore, 1959).

[1] Abbreviations used: CCD, countercurrent distribution; dimethyl-POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole.

TABLE II: Distribution of ^{3}H and ^{14}C Radioactivity of the Water-Soluble Fractions Following Countercurrent Distribution (CCD I) in the Solvent System: Ethyl Acetate–2-Butanol–0.1% Ammonium Hydroxide (425:75:500 ml). Partition Coefficients (K) Given Here are Approximate because of the Broad Peaks Obtained.

	Perfusion 1			Perfusion 2			Perfusion 3 and 4		
Component	Counts/min	%	K (n = 24)[a]	Counts/min	%	K (n = 50)[a]	Counts/min	%	K (n = 50)[a]
A	1372	47	0.05	4382	53	0.05	16,212	85	0.09
B	116	4	0.28	1600	19	0.46	1,008	5	0.39
C	214	7	3.0	920	11	2.5	378	2	2.1
D	1227	42	19.0	1380	17	48.0	1,470	8	48.0

[a] n = number of transfers.

Results

The recovery of perfused radioactivity in placental extract and perfusate and the distribution of the radioactivity between ether-soluble and water-soluble phases are indicated in Table I. The recovery of starting [^{14}C]- or [^{3}H]estrogen ranged from 82 to 90%. Following perfusion with [^{3}H]estrone [^{35}S]sulfate, 98.8–99% of the recovered ^{3}H radioactivity was in the ether-soluble fraction with only 1.0–1.2% ^{3}H radioactivity presumably still conjugated and in the water-soluble fraction. A similar distribution was found when the starting estrogen was in the free form.

Characterization of [^{3}H]Estrone [^{35}S]Sulfate and [^{3}H]Estradiol [^{35}S]Sulfate Component of Water-Soluble Radioactivity. The water-soluble fractions from the corresponding placental extracts and perfusates of the four perfusions were separately combined. Each of these fractions was then partitioned in a 24- or 50-transfer countercurrent distribution (I) in the system: ethyl acetate–2-butanol–0.1% ammonium hydroxide (425:75:500 ml). Four distinct peaks of radioactivity were obtained following each partition as indicated in Table II. In the perfusions with [^{3}H]estrone [^{35}S]sulfate, perfusions 1 and 2, component B contained superimposed peaks of ^{3}H and ^{35}S radioactivity. The ratio of ^{3}H:^{35}S in the six tubes around the peak tubes of component B was 0.31. An aliquot of component B from perfusion 2 was carried through the Brown procedure including acid hydrolysis, solvent partition, methylation, and chromatography on alumina. It was demonstrated that 89% of the tritium radioactivity was associated with estrone and 11% with estradiol. Another aliquot of component B was hydrolyzed with phenol-sulfatase from limpets; 85% hydrolysis of the tritiated material resulted. The presence of 0.12 M potassium phosphate in the incubation mixture completely inhibited the enzymatic hydrolysis of another aliquot of this unknown material. Standard estrone sulfate was 95% hydrolyzed under the conditions used. No glucuronidase activity was present in the enzyme preparation which was prepared by Sigma.

With this evidence indicating that component B was a mixture of [^{3}H]estrone sulfate and [^{3}H]estradiol sulfate, another aliquot of component B was redistributed in a 24-transfer countercurrent distribution (II) in the same solvent system following the addition of 300 μg of carrier estrone 3-sulfate. It has previously been shown that estrone 3-sulfate and estradiol 3-sulfate have similar partition coefficients in this system (Diczfalusy *et al.*, 1961a). The contents of the even numbered tubes were counted for ^{3}H and ^{35}S radioactivity; the contents of the odd numbered tubes were carried through acid hydrolysis, solvent partition, methylation, chromatography on alumina, and measurement of ^{3}H radioactivity and Kober reaction. It was possible in this fashion to demonstrate that distribution curves and partition coefficients were similar for carrier estrone 3-sulfate (K = 0.29), total ^{3}H radioactivity (K = 0.28), ^{3}H radioactivity associated with estrone (K = 0.28), ^{3}H radioactivity associated with estradiol (K = 0.27), and total ^{35}S radioactivity (K = 0.28). Once again, the ^{3}H radioactivity was associated 89% with estrone and 11% with estradiol.

These results warrant the tentative identification of component B as a combination of [^{3}H]estrone [^{35}S]-sulfate and [^{3}H]estradiol [^{35}S]sulfate. The ratio of ^{35}S: total ^{3}H over the peak tubes was 0.34–0.37 and the ratio of ^{35}S:[^{3}H]estrone was 0.37–0.40 with a ^{35}S:^{3}H ratio approximately three times greater than that of the initial estrone ^{3}H:^{35}S.

Definitive characterization of components A, C, and D was not accomplished. Component A contained a mixture of very polar material, and conjugated estrone and estradiol.

Characterization of the Ether-Soluble Radioactivity. As noted in Table I, the ether soluble fractions contained 99% of the recovered radioactivity. To obtain information concerning the nature and amount of the estrogens in the ether-soluble fraction of placentas and perfusates, aliquots with added estrone, estradiol, and estriol carriers were submitted to the complete Brown procedure, including solvent partition, methylation, chromatography on alumina, radioactivity detection, and Kober procedure. The bulk of the radioactivity followed the Kober chromogens of carrier estrone and

TABLE III: Per Cent Distribution of the ^{3}H and ^{14}C Radioactivity in Aliquots of the Ether-Soluble Fractions of Placental Extracts and Perfusates Following the Brown Procedure.

	Perfusion 1		Perfusion 2		Perfusion 3		Perfusion 4		
Fraction	Placenta (45.7)[a]	Perfusate (54.3)	Placenta (37.6)	Perfusate (62.4)	Placenta (56.9)	Perfusate (43.1)	Placenta (34.1)	Perfusate (65.9)	Average[b]
Estrone	40.0	39.9	74.1	39.8	31.7	63.4	49.3	66.8	49.7
Estradiol-17β	53.6	59.0	25.1	56.1	67.5	34.3	49.7	28.4	47.4
"Polar metabolite"	6.4	1.1	0.8	4.1	0.8	2.3	1.0	4.8	2.9

[a] Per cent of recovered ether-soluble radioactivity. [b] For combined placenta and perfusate radioactivity.

estradiol with percentage distribution as indicated in Table III. An indication from the Brown procedure of the presence of additional estrogens was the finding of an increased yield (8%) of radioactivity upon backwashing the water (estriol phase) with benzene–petroleum ether. This suggested compounds with polarity between that of estradiol and estriol. Furthermore, the radioactivity of the polar fractions which behaved like estriol on partition between benzene–petroleum ether and 2% ethanol–water did not behave like estriol after methylation, being eluted from the alumina column with benzene containing 1.4% ethanol instead of 5% ethanol as is the case with authentic free methylestriol under our experimental conditions. This suggested a compound of different polarity from estriol. No appreciable radioactivity accompanied the carrier estriol through the derivative formation and chromatography.

The ether-soluble fractions of the corresponding placental extracts and perfusates of the four perfusions were combined and then separately submitted to 99-transfer countercurrent distributions (III) in the solvent system: methanol–water–chloroform–carbon tetrachloride (350:150:200:300 ml), after addition of estrone and estradiol carriers. The results of the distributions are indicated in Table IV showing the partition coefficients of the ^{3}H and ^{14}C radioactivity and of the carrier estrogen. The distribution curves for ^{3}H and ^{14}C in the estrone and estradiol areas coincided with the distribution curves of the standard estrone and estradiol. A discrepancy between the partition coefficients of the polar component of ^{3}H and ^{14}C radioactivity and of standard estriol was noted. A comparison of the data in Tables III and IV reveals that the average distribution of ^{3}H and ^{14}C radioactivity in the estrone, estradiol, and "polar metabolite" fractions following countercurrent distribution and the Brown procedure is similar.

Because radioactive estrone and estradiol had been added to the initial perfusing fluid and because of the previously demonstrated interconversion of these two estrogens in perfused placentas (Levitz *et al.*, 1956; Troen, 1961), it was not felt that additional evidence for the identification for these two components was necessary. The nature of the other radioactive-free phenolic metabolites was studied.

Identification of [^{3}H]6-Hydroxyestradiol-17β. The contents of tubes 78–96 of distribution III from perfusions 1 and 2, which separated the polar radioactivity from estrone and estradiol in the ether-soluble fractions, were combined (41,000 counts/min). Aliquots of this

TABLE IV: Partition Coefficients (K) and Per Cent Distribution of ^{3}H and ^{14}C Radioactivity from the Ether-Soluble Fraction of Placental Perfusions Compared with the Partition Coefficients of Authentic Estrone, Estradiol-17β, and Estriol after 99 Transfers in the System: Methanol–Water–Chloroform–Carbon Tetrachloride (350:150:200:300 ml).

	K					Distribution (%)				
	Perfusion				Carrier[a]	Perfusion				
Fraction	1	2	3	4		1	2	3	4	Average
Estrone	0.20	0.20	0.15	0.21	0.20	44.5	53.0	41.0	58.4	49.2
Estradiol-17β	0.98	0.98	0.90	0.98	0.98	53.2	44.0	57.8	38.0	48.3
"Estriol fraction"	7.25	7.25	7.00	7.25	5.60	2.3	3.0	1.2	3.6	2.5

[a] As determined in CCD of perfusion 2.

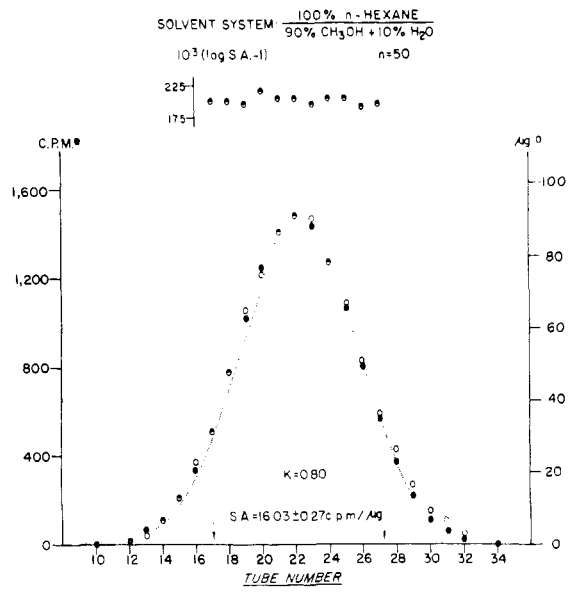

FIGURE 1: Countercurrent distribution of [^{3}H]3-methyl-6α,17β-diacetoxyestradiol prepared from [^{3}H]6α-OH-estradiol-17β plus carrier. ● = radioactivity in counts per minute; ○ = Kober chromogen in micrograms; dotted line is theoretical distribution curve.

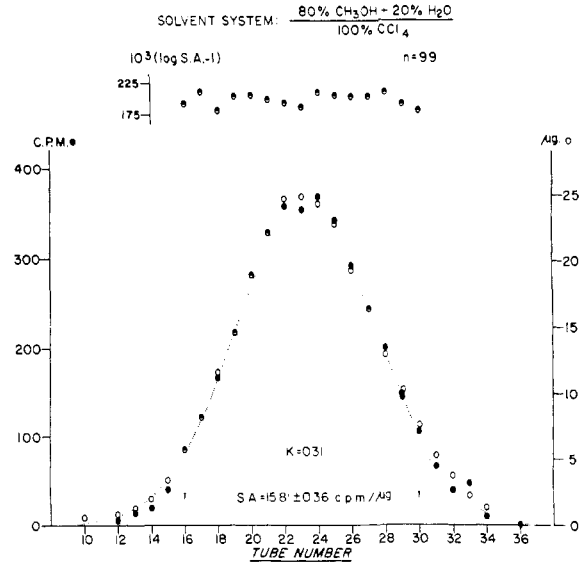

FIGURE 2: Countercurrent distribution of [^{3}H]3-methyl-6-ketoestrone prepared from [^{3}H]6α-hydroxyestradiol-17β plus carrier. Symbols as in Figure 1.

were separately subjected to 50-transfer countercurrent distributions (IV and V) in the solvent systems: (1) cyclohexane–ethyl acetate–ethanol–water (250:250:250:250 ml), (2) methanol–water–chloroform–carbon tetrachloride (350:150:400:100 ml). The ^{3}H radioactivity and the standard 6α-hydroxyestradiol-17β had identical partition coefficients in each of these solvent systems (CCD IV, K = 0.37; CCD V, K = 1.20). Accordingly, the remainder of the polar material of CCD III containing 27,600 counts/min was diluted with 1627 μg of 6α-hydroxyestradiol-17β carrier and methylated. The methyl derivative obtained (6α-hydroxy-3-methylestradiol-17β) was subjected to a 99-transfer countercurrent distribution (VI) in the solvent system: methanol–water–carbon tetrachloride (200:300:500 ml). Analysis of the distribution of radioactivity and of carrier estrogen showed both curves to have similarly shaped and located peaks, with identical partition coefficients (K = 1.21). Analysis of the specific activity of the individual tubes within $\pm 2.0\theta$ tubes of the peak indicated a specific activity of 15.39 ± 1.00 counts/min per μg (coefficient of variation 6.5%). The slope of the line relating specific activity to tube number did not deviate from zero ($P > 0.60$).

A second derivative was prepared from countercurrent distribution VI using the remaining contents of tubes 45–65. This material, containing a methyl derivative of the original compound and carrier, was acetylated. The acetylated product (13,072 counts/min and 770 μg of 3-methyl-6α,17β-diacetoxyestradiol) was partitioned in a 50-transfer countercurrent distribution (VII) in the solvent system: hexane–methanol–water (500:450:50 ml). The results of the distribution and a plot of the logarithm of the specific activity against tube number are shown in Figure 1.

Figure 1 shows that following acetylation of the methyl derivative, identity throughout the distribution curve between tritium radioactivity and carrier estrogen was obtained (K = 0.80). Analysis of the specific activity over the peak tubes revealed a value of 16.03 ± 0.27 counts/min per μg (coefficient of variation 1.68%) over the span of $\pm 1.5\theta$ tubes from the peak tube. The slope of the line relating specific activity to tube number did not deviate from zero ($P > 0.40$).

The remaining contents of tubes 13–31 of the countercurrent distribution VII containing the acetylated and methylated derivative of the unknown radioactivity and carrier 6α-hydroxyestradiol were then combined (6538 counts/min and 422 μg). This was deacetylated and then oxidized. The product of this procedure (4052 counts/min and 261 μg of 3-methyl-6-ketoestrone) was distributed in a 99-transfer countercurrent distribution (VIII) in the solvent system: methanol–water–carbon tetrachloride (400:100:500 ml). Results obtained are indicated in Figure 2. Analysis of the experimental data revealed a specific activity of 15.81 ± 0.36 counts/min per μg (coefficient of variation 2.28%) over the span of $\pm 1.5\theta$ tubes from the peak tube (K = 0.31). The slope of the line relating specific activity to tube number did not deviate from zero ($P > 0.90$). This demonstrated no separation between the labeled material and the carrier. The mean specific activities of the three derivatives sequentially formed from the unknown tritiated material diluted with 6α-hydroxyestradiol-17β carrier did not significantly differ from one another ($P > 0.40$ between CCD VI and CCD VII, and $P > 0.20$ between CCD VII and CCD VIII)

(by the Student's t test) indicating that the isolated tritiated material of placental origin was a radiochemically pure substance identical with 6α-hydroxyestradiol.

Identification of [^{14}C]*6-Hydroxyestradiol-17β.* The polar metabolite of the ether-soluble fraction from perfusions 3 and 4 was subjected to similar identification procedures as its ^{3}H counterpart from perfusions 1 and 2. The contents of tubes 78–96 of distribution III, which separated the polar radioactivity from estrone and estradiol, were combined (2752 counts/min). An aliquot containing 1376 counts/min was diluted with 148 μg of 6α-hydroxyestradiol-17β carrier and subjected to a 50-transfer countercurrent distribution (IX) in the system: methanol–water–chloroform–carbon tetrachloride (350:150:400:100 ml). Analysis of the distribution of ^{14}C radioactivity and of carrier Kober chromogen revealed both curves to have similarly shaped and located peaks with identical partition coefficients (K = 1.02). The ascending limb of the distribution curve indicated that a less polar ^{14}C metabolite overlapped the area of distribution of the authentic 6α-hydroxyestradiol-17β. The specific activity over $\pm 1.5\theta$ tubes was 7.48 ± 0.90 counts/min per μg, but only from -0.5θ to $+1.5\theta$ tubes from the peak tube was the specific activity constant. In the latter tubes, the slope of the line relating specific activity to tube number did not deviate from zero. In these tubes the specific activity was 6.98 ± 0.23 counts/min per μg.

The remaining contents of tubes 17–33 of distribution IX, 591 counts/min and 81.4 μg of 6α-hydroxyestradiol-17β, were combined with the remaining 1376 counts/min of the pool from tubes 78–96 of CCD III. The pool was methylated and the methyl derivative (1771 counts/min and 68 μg of 6α-hydroxy-3-methylestradiol-17β) was subjected to a 30-transfer countercurrent distribution (X) in the system: methanol–water–carbon tetrachloride (200:300:500 ml). The distribution of the ^{14}C radioactivity and the Kober chromogen of the carrier estrogen had identical peaks and partition coefficients (K = 1.05). The specific activity was 17.52 ± 2.68 counts/min per μg (coefficient of variation 15.75%) for $\pm 1.5\theta$ tubes from the peak tube. Again there was slight overlap of another radioactive metabolite in the ascending limb of the curve. Constant specific activity was only present from -0.5θ to $+1.5\theta$ tubes from the peak tube; in these tubes the specific activity was 15.87 ± 0.35 counts/min per μg (coefficient of variation 2.46%). The slope of the line relating specific activity to tube number did not deviate from zero ($P > 0.20$).

The remaining contents of tubes 16–24 from distribution X containing 74% of the methylated radioactivity and carrier were pooled and acetylated. The product (600 counts/min and 35 μg of 3-methyl-6α,17β-diacetoxyestradiol) was partitioned in a 30-transfer countercurrent distribution (XI) in the system: *n*-hexane–methanol–water (500:450:50 ml). There was no separation of ^{14}C radioactivity and carrier estrogen (K = 0.96). The specific activity over $\pm 1.5\theta$ tubes from the peak tube was 15.92 ± 0.46 counts/min per μg (coefficient of variation 2.89%). Radiochemical homogeneity was indicated by the constancy of the specific activity over these symmetrically distributed tubes ($P > 0.90$). Furthermore, the specific activities (where free of contamination) of the two sequential derivatives of the ^{14}C material and carrier 6α-hydroxyestradiol-17β showed no significant difference ($P > 0.80$ between CCD X and CCD XI). This evidence allows identification of [^{14}C]6α-hydroxyestradiol-17β from the perfused placenta.

Other Metabolites. The presence of radioactive metabolites with polarity intermediate between that of estradiol and estriol was suggested by the initial characterization of the free fraction by the modified Brown procedure. Identification of these metabolites will be reported later.

Discussion

Four mid-pregnancy human placentas were perfused with [^{3}H]estrone [^{35}S]sulfate (twice) and [^{14}C]estradiol-17β (twice) with the following findings: (1) evidence of an active sulfatase capability under the conditions employed; (2) evidence for a 6-hydroxylating mechanism for estrogens.

The demonstration of hydrolysis of an estrogen sulfate by the perfused human placenta is not surprising in view of the demonstration by Pulkkinen (1961) of the presence of steroid sulfatases in placental tissue. Similar results have now also been reported following *in situ* perfusion of the placenta (Mikhail *et al.*, 1963). In conjunction with our finding (Troen *et al.*, 1965) of the capability of the human placenta to sulfurylate estrogen it can be suggested that the balance present *in vivo* between sulfatase and sulfurylation activity may have a significant role in the physiologic activity of the human placenta. For example, despite the known ability of fetal tissue to sulfurylate estrogens (Diczfalusy *et al.*, 1961a; Levitz *et al.*, 1961) and the preponderance of conjugated estrogens in cord blood (Troen *et al.*, 1961), the bulk of estrogen in placental tissue is in the free form (Diczfalusy *et al.*, 1961b). However, the estrogen apparently coming from the uterus or placenta as found in the uterine vein or retroplacental blood, respectively, includes a high percentage (Klausner and Ryan, 1964) of conjugated estrogens. The balance between these free and conjugated forms may be determined by the relative activity of these two enzyme capabilities of the human placenta under different conditions. This may explain our finding of approximately 1% conjugated estrogens in recovered radioactivity after *in vitro* recycling perfusion with either free or conjugated estrogen while *in vivo* studies recently reported (Mikhail *et al.*, 1963) indicate that as much as 10–20% of conjugated radioactivity perfused through umbilical vessels may be found in placental tissue in the water-soluble phase.

It had been hoped in executing these perfusion studies with double-labeled conjugated estrogen that sufficient conjugated material would be obtained after the per-

fusions to provide data concerning possible metabolic changes of the estrogen molecule before hydrolysis, as has been suggested in fetal tissue (Emerman *et al.*, 1964). However, the approximate 1% of the material remaining conjugated after our perfusions made definitive studies difficult. The evidence reported does allow a tentative identification of a mixture of estrone sulfate and estradiol sulfate with a $^{35}S:^{3}H$ ratio of 0.34 compared to the starting ratio of 0.12. The presence of other compounds containing ^{35}S with polarity similar to estrone sulfate and estradiol sulfate is a possible explanation for this enrichment. Further studies with more extensive purification will be necessary to establish this point.

The possible occurrence of 6-hydroxylation of estradiol by the perfused human placenta was suggested by the finding of a polar estriol-like metabolite which was shown not to be estriol. The formation of radioactive 6-hydroxyestradiol-17β was demonstrated after perfusions with either [^{3}H]estrone sulfate or [^{14}C]estradiol-17β. This represents the first demonstration of the capacity of placental tissue to hydroxylate phenolic steroids at C-6.

Radiochemical homogeneity with the 6α form of the standard was established. However, it is not known whether the countercurrent distribution systems used would separate the 6α and 6β forms. There was no significant change in specific activity on conversion of the 6-hydroxy tritiated compound to the 6-keto compound. This indicates that all of the tritium initially present at position 6 was removed at the time of hydroxylation. Unfortunately, the spatial configuration of the tritium on the starting [6,7-^{3}H]estrone is not known.

Comparison with the results previously reported using the same method of study with term placentas (Troen, 1961) shows the same ready interconversion of estrone and estradiol at mid-pregnancy. Preliminary results indicate that the estrone fraction from mid-pregnancy placental perfusions also contains approximately 2% of 2-methoxyestrone as previously reported for term placentas (Troen, 1961). A difference is indicated by the identification of the polar metabolite of estrone–estradiol in mid-pregnancy placentas as being 6-hydroxyestradiol (2.1–2.3% of radioactivity recovered in the ether-soluble fraction) in contrast to the finding of predominantly estriol in the polar fraction from perfused term placentas (Troen, 1961). Countercurrent distribution of the methyl derivative as used in both identifications readily distinguishes these two estrogens. Cedard *et al.* (1961) has also reported conversion of estradiol to estriol by perfused term placentas. The percentage recoveries in our perfusions with added human chorionic gonadotropin were similar for estriol as a metabolite of estradiol in term placentas and for 6-hydroxyestradiol as a metabolite in mid-pregnancy placentas (Troen, 1961). The significance of this for the endocrine functions of the human placenta remains to be determined.

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