Perfusion Studies of the Human Placenta. IV. Conjugation of Estriol-16-^{14}C*

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ABSTRACT: Two placentas from the fifth month of normal pregnancy were perfused *in vitro* with estriol-16-^{14}C. Analysis of the radioactivity recovered from the perfusates and placental extract revealed about 1% of water-soluble material. A number of components of this fraction were separated. ^{14}C-Estriol 3-sulfate was identified. Another ^{14}C-estriol conjugate was tentatively identified as an estriol glucosiduronate.

It has been demonstrated that large amounts of estrogens circulating between the fetus and the placenta in human pregnancy are present in the conjugated form (Troen *et al.*, 1961). Similarly, a significant proportion of estrogens in retroplacental blood has been shown to be conjugated (Klausner and Ryan, 1964). Demonstration of the capacity of fetal tissues to conjugate estrogens was, therefore, an important step in clarifying estrogen metabolism during pregnancy (Diczfalusy *et al.*, 1961; Levitz *et al.*, 1961). The possible role of the human placenta in the conjugation of estrogens being carried to the fetus or to the mother has not yet been clarified, however.

The present study was undertaken to determine the capacity of human placental tissue to conjugate estrogens. Estriol-16-^{14}C was perfused through placentas obtained from mid-pregnancy and the conversion of free estriol-^{14}C to conjugated estriol-^{14}C was determined. The results demonstrate that under the experimental conditions employed human placental tissue can form conjugates of estriol.

Experimental Procedures

Perfusion and Extraction Procedures. Two intact placentas (A and B) 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. As before, recycling perfusions were carried out for 8 hr. Estriol-16-^{14}C (1.375 μcuries) and human chorionic gonadotropin (20,000 IU/l.) were added to each hourly change of perfusing fluid. The estriol-16-^{14}C (specific activity 22 μcuries/mg) was synthesized and kindly supplied by Dr. M. Levitz of New York University (Levitz, 1963). The estriol-^{14}C was shown to be over 99% radiochemically pure by countercurrent distribution. As would have been expected from the method of synthesis, there was no detectable conjugated estriol-^{14}C in the starting material.

The perfusates from each placenta were pooled and reduced in volume by evaporation at reduced pressure. The placentas were minced 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 method followed was similar to that previously used for extraction of conjugated estriol from cord blood (Troen *et al.*, 1961). The ethanolic extracts were evaporated and the residue was partitioned between 70% methanol and petroleum ether (bp 30–60°). The methanol phase was separated and evaporated at reduced pressure. The residue was taken up in distilled water which was extracted six to nine times with one-half volume of peroxide-free ether until no additional radioactivity was present in the ether extract. The ether extracts were combined and set aside for analysis of "free estrogens." This will be the subject of a separate report (M. de Miquel and P. Troen, in preparation).

The aqueous phase, presumably containing conjugated estrogens, was converted to a 70% methanol solution and kept at −15° for 48 hr. The precipitate was removed by centrifugation. The methanol was evaporated and the residue was partitioned by a 24-transfer countercurrent distribution in the solvent system: 1-butanol–0.1 N sodium hydroxide (500:500 ml). Tubes 0–14 and 15–24 from these distributions were separately combined and designated as the alkali-soluble and the butanol-soluble fractions, respectively.

Countercurrent Distributions. Countercurrent distributions (CCD)[1] were performed using an all-glass

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[1] Abbreviation used: CCD, countercurrent distribution.

TABLE I: Distribution of Radioactivity (counts per minute) through the Extraction and Fractionation Procedure.

	Placenta A	Perfusate A	Placenta B	Perfusate B
Starting estriol-16-^{14}C	5,566,000		5,566,000	
Ethanol extract				
Petroleum ether	21,000	143	16,150	—
70% Methanol	827,460	4,525,560	737,995	3,556,160
70% Methanol fraction				
Ether	793,500	4,492,610	720,000	3,492,180
Water	32,440	23,220	17,887	22,680
Water phase				
Alkali soluble	17,193	14,756	12,521	10,886
Butanol soluble	15,247	6,944	5,366	11,794

100-tube automatic Craig-Post apparatus. Calculations of partition coefficients and theoretical distributions were made according to Weisiger (1954). Statistical analysis of the experimental data relating logarithms of the specific activities to the tube number as a criterion of radiochemical purity was performed according to Baggett and Engel (1957).

Radioactivity Measurement. Measurement of radioactivity was performed using a thin-window Geiger flow detector with an efficiency of approximately 25%. In general, planchets were counted at infinite thinness; where necessary because of self-absorption, correction to infinite thinness was done. Sufficient counts were accumulated for the standard error to be 2–3%.

Determination of Estriol. In most instances, a direct Kober reaction, using the technique developed by Brown (1955), could be applied. In critical instances, the material was hydrolyzed and processed according to the full Brown method including solvent partition, methylation, alumina chromatography, and the Kober reaction.

After countercurrent distributions, aliquots for the Kober reaction were usually taken from individual tubes of the distribution. Occasionally, amounts as low as 0.15–0.20 μg were determined at the end tubes of the distribution curves. In such instances, half-volumes of Kober reagent and 20-mm long cuvets were used for spectrophotometry. This means of increasing the sensitivity of the method fourfold was possible because of the very clean extracts being studied. The accuracy and validity of this method has previously been confirmed (Diczfalusy and Magnusson, 1958; de Miquel, 1961).

Preparation of Derivatives. Acetylation and methylation were carried out as previously described (Troen *et al.*, 1961).

Hydrolysis. Acid hydrolysis was carried out using the conditions of Brown (1955). Glucuronidase hydrolysis and solvolysis were carried out as previously described (Troen *et al.*, 1961). Sulfatase hydrolysis was accomplished with Type III (limpet) sulfatase of Sigma, incubating for 18 hr at 37° with an enzyme concentration of 0.5 mg/ml in a pH 5.0 sodium acetate buffer.

Standards. Authentic estriol 3-sulfate was obtained through the courtesy of Dipl. Eng. H. Fex, AB Leo, Hälsingborg, Sweden. Reference compound conjugate B was isolated from fluids of newborn infants (Troen *et al.*, 1961).

Results

The placental extracts and corresponding perfusate extracts were processed separately. The distribution of radioactivity through the extraction and fractionation process is indicated in Table I. Recovery of the original radioactivity was 97% for perfusion A and 77% for perfusion B. The butanol-soluble and alkali-soluble fractions of the water phase were analyzed further.

Butanol-Soluble Fraction. Identification of ^{14}C-estriol 3-Sulfate. The water-soluble fraction containing approximately 1% of the recovered radioactivity was put through a 24-transfer countercurrent distribution in the solvent system: 1-butanol–0.1 N sodium hydroxide (500:500 ml) (CCD I). In each placental and perfusate extract, a radioactive component was obtained with a partition coefficient of 4.4. The partition coefficient of standard estriol 3-sulfate in this system is 4.6. This component, referred to as the butanol-soluble fraction, contained approximately 42% of the radioactivity of the water-soluble fraction.

The contents of tubes 15–24 of CCD I from placenta A representing 95% of the total radioactivity of the butanol-soluble fraction were combined and the residue of 14,000 counts/min after evaporation of the solvents was submitted to a 24-transfer countercurrent distribution in the solvent system: 1-butanol–ethyl acetate–0.2% ammonium hydroxide (250:250:500 ml). In this second distribution (CCD II) 80% of the total radioactivity showed a partition coefficient identical with that of the estriol 3-sulfate in this system (K = 2.5). A second radioactive peak, smaller and less polar, was noted with a partition coefficient of 32, corresponding

to the K for free estriol in this system. This second component was considered to be a small residual of the starting ^{14}C-estriol not removed from the water-soluble fraction. The contents of tubes 12–20 of the CCD II containing the ^{14}C-estriol 3-sulfate like material were pooled; to an aliquot containing 5010 counts/min, 390 μg of carrier estriol 3-sulfate was added (specific activity 12.84 counts/min per μg). After evaporation of the solvent, the residue was subjected to a 24-transfer countercurrent distribution in the same solvent system (CCD III). In this third countercurrent distribution a single component was found. The distribution curves of both radiochemical material and carrier estrogen were represented by a single theoretical curve with identical partition coefficients (K = 2.5). The specific activity (counts/minute per microgram of carrier estrogen) was 12.5 ± 0.14 (coefficient of variation 1%) over a span of $\pm 1.5\theta$ tubes at the peak of the distribution. Analysis of the data relating the logarithm of specific activities with the tube number revealed a straight line with a slope not significantly different from zero ($P > 0.9$).

The contents of tubes 12–22 of CCD III were pooled. The pool contained 4600 counts/min and 364 μg of carrier, specific activity 12.63 counts/min per μg. This was redistributed in a 50-transfer countercurrent distribution in a fourth solvent system: 2-butanol–water (500:500 ml) (CCD IV). The partition coefficients in this system were once again identical for the radioactive material and the carrier estriol 3-sulfate (K = 1.8). The specific activity of a pool of the peak tubes (numbers 30–34) of this distribution was 12.6 counts/min per μg, not significantly different from the specific activity obtained in CCD III.

These results provided initial evidence for the identity and radiochemical homogeneity of the presumed ^{14}C-estriol 3-sulfate. Further data were sought by derivative formation. An aliquot from CCD IV containing 1682 counts/min and 134 μg of carrier estriol 3-sulfate was acetylated and then subjected to a 99-transfer countercurrent distribution in the solvent system: 2-butanol–*n*-hexane–0.5% sodium chloride (400:100:500 ml). Identical partition coefficients (K = 1.9) were obtained for the radioactivity and the Kober chromogen of the derivative, estriol 3-sulfate 16,17-diacetate (CCD V). The specific activity of 12.3 counts/min per μg obtained from a pool of the central tubes (numbers 17–21) of the distribution was not significantly different from the specific activity values obtained in the previous distributions.

To another aliquot from CCD IV containing 1682 counts/min and 134 μg of carrier estriol 3-sulfate (specific activity, 12.55 counts/min per μg) was added another 110 μg of carrier estriol 3-sulfate (final specific activity 6.89 counts/min per μg). This material was acetylated. The acetylation product, estriol 3-sulfate 16,17-diacetate, was then solvolyzed. The final product, estriol 16,17-diacetate, was put through a 24-transfer countercurrent distribution in the solvent system: benzene–*n*-hexane–methanol–water (150:350:250:250 ml) (CCD VI). The partition coefficients for the radioactivity and the Kober chromogenicity were identical (K = 2.9). The specific activity over $\pm 1.0\theta$ tubes at the peak of the distribution was 6.88 ± 0.06 counts/min per μg (coefficient of variation 0.87%). Analysis of the data showed that the calculated slope of the line relating specific activity to tube number was not significantly different from zero ($P > 0.8$). Furthermore, when the specific activities were corrected for added carrier, there was no significant difference between the mean specific activities of CCD III and CCD VI ($P > 0.2$ by Student's t test).

Hydrolytic methods were also used to demonstrate the estriol moiety of the conjugate using an aliquot (421 counts/min and 33.5 μg of carrier estriol 3-sulfate (specific activity 12.5 counts/min per μg)) from the peak tubes of CCD IV. After either acid hydrolysis or phenolsulfatase hydrolysis, the free radioactivity and carrier estriol thus produced were subjected to solvent partition, derivative formation (methylation), and chromatography. The specific activities of the product ^{14}C-estriol 3-methyl ether were unchanged from the starting specific activity of the ^{14}C-estriol 3-sulfate: 12.2 counts/min per μg after acid hydrolysis and 12.7 counts/min per μg after phenolsulfatase hydrolysis.

To demonstrate the sulfate nature of the conjugation of the unknown, enzymic hydrolysis was performed using a phenolsulfatase prepared from limpets (Sigma). Previous treatment of the enzyme preparation by the supplier had rendered it substantially free of β-glucuronidase activity and this was confirmed by the finding of no hydrolysis of standard estriol monoglucosiduronate. Under the incubation conditions used, 100% hydrolysis of standard estriol 3-sulfate was achieved. Incubation of the presumed ^{14}C-estriol 3-sulfate with phenolsulfatase resulted in 88% hydrolysis. The presence of 0.12 M phosphate, an inhibitor of sulfatase activity, in the incubation mixture completely inhibited the enzymic hydrolysis.

Alkali-Soluble Fraction. The initial separation of the water-soluble fraction by countercurrent distribution in the butanol–alkali system (CCD I) yielded approximately 58% of the radioactivity (17,200 counts/min) in tubes 0–12, referred to here as the alkali-soluble fraction. In the initial 24-transfer distribution no satisfactory separation of this fraction into individual components was obtained. The material was therefore redistributed in a 200-transfer countercurrent distribution in the same solvent system: 1-butanol–0.1 N sodium hydroxide (500:500 ml). Seventy-one percent of the radioactivity (12,200 counts/min) was present in a broad component (I) with a partition coefficient of approximately 0.08. Sixteen percent of the radioactivity (2750 counts/min) was present in another component (II) with a partition coefficient of 0.32. The remaining radioactivity was diffusely spread out in poorly defined areas.

Characterization of Component I. Component I (tubes 0–30) of the 200-transfer butanol–alkali distribution was redistributed for 99 transfers in the system: ethyl acetate–1-butanol–0.2% ammonium hydroxide (250:250:500 ml). Two distinct peaks of approximately equal amounts were obtained with partition coefficients

TABLE II: Comparison of Partition Coefficients of the ^{14}C-Estriol Conjugate Portion of Component II of the Alkali-Soluble Fraction and of Partition Coefficients of Other Estriol Conjugates.

Solvent System (ml)	Unknown	"Conjugate B"	"Conjugate A"	"Conjugate C"	Estriol-16(?17)-monogluc.[a]	Estriol-3-monogluc.[b,c]	Estriol 3-Sulfate[a]
1-Butanol–0.1 N sodium hydroxide (500:500)	0.32	0.33	0.12	...	0.06	0.45	4.6
1-Butanol–ethyl acetate–0.2% ammonium hydroxide (250:250:500)	0.06	0.06	0.06	0.06	1.1	0.09	2.1
1-Butanol–*t*-butyl alcohol–2 N ammonium hydroxide (375:125:500)	0.56	0.56	0.18	0.67	0.69	0.18	2.6

[a] Troen *et al.*, 1961. [b] Beling, 1963. [c] C. G. Beling, personal communication.

of 0.05 (component Ia) and 2.0 (component Ib), respectively.

To obtain an indication of whether components Ia and Ib were conjugated compounds, they were separately subjected to acid hydrolysis and the hydrolysis product was redistributed in the ethyl acetate–butanol–0.2% ammonium hydroxide system noted above. The partition coefficient of component Ia was essentially unchanged by acid hydrolysis; that of component Ib was markedly increased to 24. This suggests that component Ia is not a conjugate. Component Ib may be a conjugate, according to the hydrolysis results, but the presence of significant amounts of estriol was excluded by the results of the Brown procedure.

Characterization of Component II. Probable Estriol Glucosiduronate. Preliminary evidence that component II may contain a conjugate of ^{14}C-estriol was obtained when 80% of the radioactive product of acid hydrolysis behaved like carrier estriol when carried through solvent partition, methylation, and alumina column chromatography. The remainder of the hydrolysis product of compound II did not behave like estriol. To characterize further the 80% of the component II which appeared to be a conjugate of estriol, component II was examined by countercurrent distributions with the contents of each tube being analyzed by the complete Brown procedure. In this matter, distribution curves were obtained of the radioactivity associated with estriol in each tube. The results in three solvent systems are shown in Table II. It can be seen that the partition coefficients agree with those of an estriol conjugate identified in fluids of newborn infants by Troen *et al.* (1961) and referred to by them as conjugate B. Evidence that conjugate B may be a polyglucosiduronate of estriol has been presented elsewhere (Troen *et al.*, 1961). In the first two solvent systems in Table II, no carrier conjugate B was added. In the third solvent system, carrier conjugate B from urine of newborn infants was added. When the complete Brown procedure was carried out on each tube, there was excellent coincidence of the distribution curves of radioactivity and Kober chromogen of the estriol 3-methyl ether so obtained. Too little material was available to allow calculation of specific activities for individual tubes of the distribution. It should be noted that although the partition coefficients for radioactivity and carrier conjugate B were identical in the third solvent system, the value obtained was higher than that previously recorded (Troen *et al.*, 1961), possibly due to altered environmental conditions. In Table II are also listed the reported partition coefficients in these three solvent systems of other estriol conjugates. The latter can be excluded as being identical with the ^{14}C-estriol conjugate present in component II. The data in Table II warrant a tentative identification of conjugate B (an estriol glucosiduronate) as being the major radioactive constituent of component II of the alkali-soluble fraction obtained from placentas perfused with estriol-16-^{14}C.

Discussion

The data presented permit an identification of ^{14}C-estriol 3-sulfate as a conjugation product of estriol-16-^{14}C perfused through human placentas obtained from about the 20th week of normal pregnancy. The evidence for this identification may be summarized as follows: (1) partition coefficients in three sequential solvent systems; constant specific activity; (2) partition coefficients of two sequential derivatives; constant specific activity; (3) release of ^{14}C-estriol after acid or enzymic hydrolysis; constant specific activity; (4) hydrolysis with phenolsulfatase; inhibition of hydrolysis with phosphate.

Another compound has been found which is tentatively identified as an estriol conjugate, conjugate B, recently described by Troen *et al.* (1961) who have presented evidence to indicate that conjugate B is probably a di- or triglucosiduronate of estriol. It appears, therefore, that conjugation of estriol-16-^{14}C as a glucosiduronate, as well as a sulfate, took place during placental perfusion. These results appear to be the first report

of estrogen conjugation by the human placenta.

Other metabolites of perfused ^{14}C-estriol were detected in the water-soluble fraction. Their nature is unknown but they appear to include an unconjugated, very polar metabolite (component Ia) and a possibly conjugated metabolite (component Ib). Component Ia may be the same as the unidentified, very polar, unconjugated metabolite of estriol reported by Mikhail *et al.* (1963) following perfusion of fetuses with ^{14}C-estriol. The possibility that this is a 6-hydroxylated metabolite of estriol is suggested by the recent finding of 6-hydroxylation of estrogen by the perfused placenta (Alonso and Troen, 1965).

The possibility of estrogen sulfurylation in the human placenta was studied by Levitz *et al.* (1960) following their positive result with guinea pig placenta. However, they found no indication of sulfurylation by a perfused 3-month human placenta and because of insufficient material could not identify any estrogen sulfate following human placental tissue incubation (Levitz *et al.*, 1961).

That the estriol conjugates formed during *in vitro* perfusion of the placenta may be of physiologic significance is indicated by their presence *in vivo;* estriol 3-sulfate is the major estriol conjugate of human cord blood, and conjugate B, found in small amounts in cord blood, is present in large amounts in urine of newborn infants (Troen *et al.*, 1961). However, fetal tissues have also been shown to be highly active in estrogen sulfurylation (Diczfalusy *et al.*, 1961) and formation of an estriol glucosiduronide by fetal tissue has been reported (Engel *et al.*, 1962).

The yield of conjugated estrogens from *in vitro* placental perfusion was very small (approximately 1%). However, it is possible that the very small yield may be related to the recycling technique used. This may allow a relatively greater effect of hydrolytic mechanisms than of conjugate formation. Indeed, estrogen sulfatase activity has been demonstrated by Pulkkinen (1961) in incubation studies and has been confirmed in our perfusion studies with mid-pregnancy placentas (Alonso and Troen, 1965).

The presence of sulfatase activity in placental tissue and the very small yield of ^{14}C-estriol 3-sulfate following perfusion raise the question of whether the conjugate obtained is the product of enzymatic sulfurylation (with a sulfate-activating system and a sulfokinase) or the result of a transferase action of the sulfatase (Suzuki *et al.*, 1957). Additional studies would be necessary to resolve this question.

The juxtaposition of two organs, the placenta and fetus, so directly concerned with estrogen metabolism (*cf.* the review of Diczfalusy and Troen, 1961), suggests that analysis of the nature and amount of free and conjugated estriol circulating between these organs in the umbilical artery and vein, respectively, might provide more direct evidence for the relative role of each. The results of these studies are the subject of a separate report (P. Troen, in preparation).

The demonstration of formation and hydrolysis of a sulfate of estriol may have a counterpart in steroid glucosiduronate formation by the placenta. β-Glucuronidase activity has been shown in placental tissue (Fishman and Anlyan, 1947) and in placental perfusate (P. Troen, unpublished observations), and data have been reported indicating glucosiduronate formation of endogenously formed Porter–Silber chromogens by the perfused placenta (Troen, 1961). The physiologic significance of these processes remains to be demonstrated.

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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*

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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.

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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