PROGESTERONE METABOLISM IN THE PREVIABLE HUMAN FETUS

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by

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ABBREVIATIONS AND TRIVIAL NAMES

The following abbreviations and trivial names are used in this thesis.

cholesterol	cholesta-5-en-3β-ol
progesterone	pregn-4-ene-3,20-dione
20α-dihydroprogesterone	20α-hydroxypregn-4-en-3-one
20β-dihydroprogesterone	20β-hydroxypregn-4-en-3-one
pregnanolone	3α-hydroxy-5β-pregnan-20-one
3β-hydroxy-5α-pregnan-20-one	3β-hydroxy-5α-pregnan-20-one
pregnanediol	3α,20α-dihydroxy-5β-pregnane
3α,20β-dihydroxy-5β-pregnane	3α,20β-dihydroxy-5β-pregnane
3β,20β-dihydroxy-5α-pregnane	3β,20β-dihydroxy-5α-pregnane
pregnanedione	5β-pregnan-3,20-dione
6β-hydroxyprogesterone	6β-hydroxypregn-4-ene-3,20-dione
16α-hydroxyprogesterone	16α-hydroxypregn-4-ene-3,20-dione
17α-hydroxyprogesterone	17α-hydroxypregn-4-ene-3,20-dione
pregnenolone	3β-hydroxypregn-5-en-20-one
17α-hydroxypregnenolone	3β,17α-dihydroxypregn-5-en-20-one
deoxycorticosterone	21-hydroxypregn-4-ene-3,20-dione
corticosterone	11β,21-dihydroxypregn-4-ene-3,20-dione
16α,17α-dihydroxyprogesterone	16α,17α-dihydroxypregn-4-ene-3,20-dione
17α,20α-dihydroxyprogesterone	17α,20α-dihydroxypregn-4-en-3-one
17α,20β-dihydroxyprogesterone	17α,20β-dihydroxypregn-4-en-3-one
hydrocorticone (cortisol)	11β,17α,21-trihydroxypregn-4-ene-3,20-dione
aldosterone	11β,21-dihydroxypregn-4-en-18-al-3,20-dione

16α-hydroxyhydrocortisone	11β,16α,17α,21-tetrahydroxyprogn-4-one-3,20-dione
18-hydroxyprogesterone	18-hydroxyprogn-4-one-3,20-dione
18-hydroxycorticosterone	11β,18,21-trihydroxyprogn-4-one-3,20-dione
18-hydroxydeoxycorticosterone	18,21-dihydroxypregn-4-one-3,20-dione
deoxycortisol	17α,21-dihydroxyprogn-4-one-3,20-dione
testosterone	17β-hydroxyandrost-4-en-3-one
androstenedione	androst-4-one-3,17-dione
16α-hydroxyandrostenedione	16α-hydroxyandrost-4-one-3,17-dione
11β-hydroxyandrostenedione	11β-hydroxyandrost-4-one-3,17-dione
dehydroisoandrosterone	3β-hydroxyandrost-5-en-17-one
androstenetriol	3β,16α,17α-trihydroxyandrost-5-ene
estriol	estra-1,3,5(10)-triene-3,16α,17β-triol
pregnanolone sulphate	20-keto-5β-pregnan-3α-yl sulphate
20-keto-5α-pregnan-3β-yl sulphate	20-keto-5α-pregnan-3β-yl sulphate
pregnenolone sulphate	20-ketopregn-5-ene-3β-yl sulphate
dehydroisoandrosterone sulphate	17-ketoandrost-5-ene-3β-yl sulphate
pregnanediol sulphate	3α,20α-dihydroxy-5β-pregnan-(3 or 20)-yl sulphate
pregnanediol glucuronide	3α,20α-dihydroxy-5β-pregnan-3α-yl-β-D-glucopyranosiduronic acid
deoxycorticosterone sulphate	3,20-diketopregn-4-en-21-yl sulphate
corticosterone sulphate	11β-hydroxy-3,20-diketopregn-4-en-21-yl sulphate

estriol sulphate	estra-1,3,5(10)-triene-16α,17β-diol-3 sulphate
estriol glucuronide	estra-1,3,5(10)-triene-3α,17β-diol-16α-yl-B-D-glucopyranosiduronic acid
ACTH	adrenocorticotrophic hormone
µg	microgram
mg	milligram
µc	microcurie
cpm	counts per minute
ml	millilitor
XL	crystal
ML	mother liquor

INTRODUCTION

During the last ten years a large number of publications have been devoted to problems of steroid metabolism in the human fetus. Much of the interest in this area was stimulated by the review article of Lanman (1) in which he re-emphasized the unique physiological and anatomical features of the fetal zone of the adrenal. He also pointed out that this structure was present in simians and more recently it has been described in the armadillo (2).

When the investigations to be described were initiated, a number of publications had described the *in vitro* conversion of various substrates to steroid metabolites by the human fetal adrenals, testes, ovaries, and liver. Our approach to the problem of steroid metabolism in the midterm human fetus was based on perfusion of the fetus with labelled precursors. To accomplish this aim we initiated a very profitable collaborative study with Professor Egon Diczfalusy at the Karolinska Hospital, Stockholm, Sweden. The perfusion of the fetuses and the initial tissue extractions were carried out in his laboratories.

This thesis will describe the methods used and the results obtained from the perfusion of intact and totally adrenalectomized previable human fetuses obtained at therapeutic abortions carried out in Stockholm. Before proceeding to a description of my own work a brief historical review will serve to orient this data in relation to what was already known about steroid metabolism in the human fetus.

The presence of neutral steroids in the blood and tissues of the human fetus at varying times throughout normal gestation has been documented (3,4,5). Similarly, neutral steroids have been found in extracts of placentae (6,7). Such findings have raised the question as to whether the steroids isolated from the fetal tissues were produced therein, formed in the placenta, or synthesized in the maternal tissues and transported to the fetus. In an attempt to answer these questions a number of investigators have been studying maternal-fetal steroid metabolism using numerous experimental approaches.

Trans placental passage of steroids has been demonstrated by the injection of labeled precursors into either the maternal or fetal compartment and the subsequent isolation of the compound or its metabolite(s) in the other compartment. Thus the passage of 4-^{14}C-progesterone from the mother to the fetus has been shown (8). An approximation of the production rate of progesterone shows that it does rise during pregnancy (9) but the contribution from the fetus is thought to be low as evidenced by the minimal fall in urinary pregnanediol when the umbilical cord is clamped (10). There has been considerable discussion about the true value of progesterone production rates, as the urinary metabolite pregnanediol used in the determination is not uniquely derived from progesterone (11). Thus secretion rates of progesterone based on urinary pregnanediol are artificially high. This is especially true for the non-pregnant state and may be important in pregnancy. The increase in

progesterone production in pregnancy is assumed to come primarily from the placenta although there is some increase in ovarian production in the first trimester (12).

The transfer of many steroids between the mother and fetus has been described (13,14). Maternal cortisol may well account for about 80% of the circulating cortisol in the fetus and this is supported by recent studies in Addisonian's maintained on prednisone (15). In these patients no cortisol was found in the maternal circulation and the trace amounts found in the fetal circulation were assumed to come from the fetal endocrine tissues.

The transfer of conjugated steroids across the placenta has also been studied. Following injection of ^{3}H-estriol-3-sulphate and ^{14}C-estriol-16-glucuronide into the amniotic fluid at midterm, ^{3}H-estriol in the free and sulphate forms and ^{14}C-estriol as the glucuronide were isolated from maternal urine (16).

Bolté et al. have demonstrated the transfer of dehydroisoandrosterone sulphate (DHAS) across the placenta both from the mother to the fetus (17) and from the fetus to the mother (18).

The role of the placenta in steroid biosynthesis at mid- and full-term has been extensively studied using both <u>in vitro</u> incubations and <u>in situ</u> perfusions. The <u>in vitro</u> production of progesterone from 1-^{14}C-acetate has been demonstrated in low yield (19) but the most efficient precursors of placental progesterone seem to be cholesterol (20) and pregnenolone (21,22).

Progesterone is transformed to a number of products in placental incubation studies. Thus 6β-hydroxyprogesterone (23),

17α-hydroxyprogesterone (24), 20α-dihydroprogesterone (25) and 16α-hydroxyprogesterone (26) have all been isolated from the incubation of term placenta preparations when the added substrate was progesterone.

The formation of testosterone or Δ^4-androstenedione by incubation of placental tissue starting with C-21 precursors has been unrewarding, thus suggesting a lack of the C_{17-20} desmolase enzyme in the placenta. When C-19 compounds such as androstenedione or testosterone are used as the precursor, rapid aromatization to estrogens will occur in placenta incubation studies.

When midterm placentas were perfused <u>in situ</u> with pregnenolone, it was efficiently converted to progesterone (27,28) but no further metabolism of the precursor could be demonstrated (29). Testosterone conversion to estradiol in the perfused midterm placenta has been demonstrated (30) as well as the conversion of dehydroisoandrosterone and androstenedione to estriol (31). When the isolated term placenta was perfused with pregnenolone it was possible to demonstrate the formation of progesterone and 6β-hydroxyprogesterone (32).

In an effort to define the biosynthetic potentialities of the human fetal adrenal, extensive studies using slices, minces, and homogenates of adrenals obtained at early, mid, or late gestation, have been carried out. When acetate was used as the substrate, adrenal slices were capable of forming androstenedione, dehydroisoandrosterone, and hydrocortisone (33).

The conversion of pregnenolone to progesterone by the fetal

adrenal is very dependent on the cofactors (34) present in the incubating medium, and on the gestational age of the fetus from which the tissue is obtained. This conversion requires two enzymes, the 3β-hydroxysteroid dehydrogenase and the Δ^5-isomerase. It has been postulated (35) that there is a lack of the 3β-hydroxysteroid dehydrogenase enzyme until late pregnancy and this is supported by the histochemical studies of the fetal adrenal (36,37). It has been shown that this enzyme is absent from the fetal zone of the cortex throughout gestation and is only found in the thin rim of true cortex after the fourth month of gestation.

Progesterone is efficiently metabolised when used in fetal adrenal incubations. There appears to be a definite pattern of products formed which changes as gestation progresses. It has been assumed that the metabolites formed reflect serial enzyme changes in the fetal adrenal. Thus the 11β-hydroxylase and 17α-hydroxylase appear by the tenth week of gestation, 21-hydroxylase later, at about the fifteenth week (38) and the 18-hydroxylase, much later, probably close to thirty weeks. Table I shows the steroids that have been isolated from fetal adrenal incubation studies when progesterone was used as the substrate. Also shown in this table are the steroids isolated from the adrenals of newborns incubated with progesterone.

The formation of conjugated steroids in fetal adrenal incubations has been studied extensively. The presence of sulfokinase in various fetal tissues has been demonstrated (46). The fetal

TABLE I

FORMATION OF NEUTRAL STEROIDS IN FETAL AND NEWBORN ADRENALS USING PROGESTERONE AS THE PRECURSOR

STEROID	REFERENCE	
	FETAL	NEWBORN
Cortisol	45	42,43,44
Corticosterone	-	42,43,44
11-deoxycortisol	-	42
11-deoxycorticosterone	40	44
16α-hydroxycortisol	41	-
17α-hydroxyprogesterone	39	44
16α-hydroxyprogesterone	40	44
16α,17α-dihydroxyprogesterone	45	-
Δ^4-androstenedione	39	44
11β-hydroxyandrostenedione	45	-

adrenal sulphurylating activity exceeds that present in the fetal liver and in the adult adrenal and liver. Sulphurylation of cortisol, corticosterone, 11-deoxycortisol and 11-deoxycorticosterone has been reported (47) using adrenals obtained from full term or premature infants. The isolation of dehydroisoandrosterone sulphate following an incubation of midterm fetal adrenal homogenate with pregnenolone has also been reported (48).

In addition to the adrenal, other tissues of the human fetus have been incubated with various steroid precursors. Fetal liver, incubated with progesterone led to the isolation of 20α-dihydroprogesterone and of a compound which was partially identified as pregnanediol (49). The fetal ovary, at least up to twenty weeks gestational age, was relatively inactive with 20α-dihydroprogesterone being the only compound identified after incubation with progesterone (50). The fetal testes on the other hand were quite active at an early age of gestation, showing especially, extensive 17α-hydroxylase and side-chain splitting activity leading to significant testosterone production. The formation of steroids from 1-^{14}C-acetate using an in vitro organ culture of human fetal testes has been studied and pregnenolone, progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone were isolated (51). Incubation studies using homogenates (52) and minces (50) of fetal testes have been carried out with 4-^{14}C-progesterone and 7α-^{3}H-pregnenolone as precursors. Table II shows the products isolated from such studies. The formation of the Δ^4-3-keto steroids, testosterone and androstenedione

TABLE II

PRODUCTS OF PROGESTERONE AND PREGNENOLONE

INCUBATION IN HUMAN FETAL TESTES

PROGESTERONE	REF.	PREGNENOLONE	REF.
16α-hydroxyprogesterone	52	Testosterone	50
17α-hydroxyprogesterone	50, 52	Androstenedione	50
20α-dihydroprogesterone	50, 52	Dehydroisoandrosterone	50
Testosterone	50, 52	Androstenetriol	50
Androstenedione	50	17α-hydroxyprogesterone	50
17α-hydroxyandrostenedione	50	17α-hydroxypregnenolone	50
20β-dihydroprogesterone	50		
Deoxycorticosterone	50		
Deoxycortisol	50		
17α, 20α-dihydroxyprogesterone	50		
17α, 20β-dihydroxyprogesterone	50		

from pregnenolone suggests that there is no lack of 3β-hydroxysteroid dehydrogenase in such in vitro studies and this is corroborated by the histochemical studies of Baillie et al. (53) in which the enzyme was found in the testes of very young fetuses.

The assessment of steroid synthesis in the fetus and placenta has been based primarily on the results of incubation or perfusion studies of individual tissues. The results of such experiments are open to reasonable criticism concerning the presence or absence of necessary cofactors in incubations and the removal or disruption of organ interreactions in perfusion studies. Thus incubation or perfusion studies of individual organs reflect only the ability of the selected tissue to metabolize the substrate under the particular conditions of that experiment. In an effort to overcome some of these objections, we became interested in the technique of perfusing the whole fetus via the umbilical vein. We hoped that such studies would give us a clearer concept of the metabolism of progesterone in the midterm previable human fetus when tissues were functioning in close to normal relationship one to the other.

Similar studies have been carried out previously for the C-18 and C-19 steroids (54,18) and the metabolic fate of free and conjugated estrogens in the fetal-placental unit has been well defined (55). Much less information is available about the fate of C-21 steroids. Perfusion of pregnenolone into midterm fetuses resulted in the isolation of dehydroisoandrosterone sulphate and pregnenolone sulphate from the adrenal (56). Using 4-^{14}C-progesterone as the precursor in perfusion

of midterm fetuses, Zander (57) has reported the isolation of 17α-hydroxyprogesterone, 6β-hydroxyprogesterone, 20α-dihydroprogesterone and 20β-dihydroprogesterone from umbilical artery blood. In the same experiment he reported the presence of pregnanediol in the liver of the perfused fetus. Zander has also carried out perfusion of a full term anencephalic fetus (58) and identified 20α-dihydroprogesterone, pregnanediol, and pregnanedione in the unconjugated fraction. The predominant steroid in the hydrolyzed "sulphate" fraction was identified as pregnanediol. The isomer, 5β-pregnane-3β,20β-diol was also detected, but all of the identifications were based on the mobilities of the metabolites on paper chromatograms, in comparison with authentic steroids.

The embryological development of the adrenal gland and its morphological characteristics help support the position of prominence held by this gland in steroid production and metabolism in the fetus. In man, the adrenal consists of two glands united together. The cortex and medulla are developmentally, histologically and may be functionally unrelated. Each part is derived from a distinct and different parent tissue (59). This discussion is concerned with the cortex which is mesodermal in origin being derived from the ventral portion of the celomic epithelium. The mesothelial buds appear in the 5-6 mm embryo and by the time the embryo is 12-15 mm these buds have separated from the parent mesothelium and form compact cellular masses lateral to the aorta. The gland grows steadily so that by the third month of gestation it is somewhat larger than the

kidney. The further growth rate is slower than that of other organs so that by birth it is about one-third the size of the kidney.

Our studies in the previable fetus are carried out between 16 and 20 weeks gestation when the fetal adrenal is relatively large in size. At this time two distinct cell types are discernible in the cortex. The outer rim of cells make up only 20% of the total cortex (1). This layer is the precursor of the adult cortex and histologically resembles the glomerulosa cell layer. It has been suggested (60) that this zone is relatively inactive physiologically as its chief product in the adult, aldosterone, is not required by the fetus in the maintenance of electrolyte homeostasis (45). Salt and water metabolism in the fetus is probably controlled by the placenta. If the suggestion that fetal pituitary ACTH is the trophic factor acting on the fetal adrenal, the discrepancy in size of the two cell layers becomes even more logical as ACTH in physiological amounts does not seem to cause much increase in aldosterone production by the adult adrenal (61).

The inner part of the cortex is referred to as the fetal or X zone. Histologically these cells are like those of the reticular zone in the adult adrenal cortex. This is the active tissue in terms of growth and accounts for the large size of the adrenal in early life. The precise trophic factor causing this rapid tissue growth is not definitely known. Many factors have been considered responsible for the growth of this tissue and include chorionic gonadotrophin (62), growth hormone (63) and estrogens (64). However,

the current concepts suggest that fetal ACTH is the trophic agent (65). In anencephalic infants with much smaller pituitaries than normal, there is parallel atrophy of the adrenal fetal zone but not of the outer adult zone. The presence of an actively growing fetal adrenal cortex plus the evidence from incubation studies of specific enzyme deficiencies in both the adrenals and placenta suggested that the fetus and its placenta function as a carefully coordinated biochemical unit with certain reactions occuring in one compartment followed by transfer via the umbilical cord circulation to the other compartment where the next sequential reactions take place.

In view of the 3β-hydroxysteroid dehydrogenase deficiency in the fetal adrenal the production of progesterone from pregnenolone was postulated as occuring in the placenta. This progesterone then is carried to the fetus and cleared by the fetal tissues as suggested by the umbilical vein-artery concentration gradient of progesterone which has been demonstrated by several workers (66,67). The capability of the fetal tissues to metabolize progesterone by hydroxylation, reduction and side chain cleavage has been demonstrated. Sulphurylation then occurs in the fetal adrenal or liver and the product returns to the placenta where hydrolysis and aromatization of the proper precursor can occur.

Thus, when our studies began it seemed necessary to investigate the metabolism of progesterone in the previable human fetus employing a more physiological approach. To approach such conditions more

closely we chose to perfuse the intact and adrenalectomized fetuses using oxygenated, heparinized adult human blood containing 4-^{14}C-progesterone. Following perfusion the tissues were extracted and metabolites isolated and identified.

METHODS

SELECTION

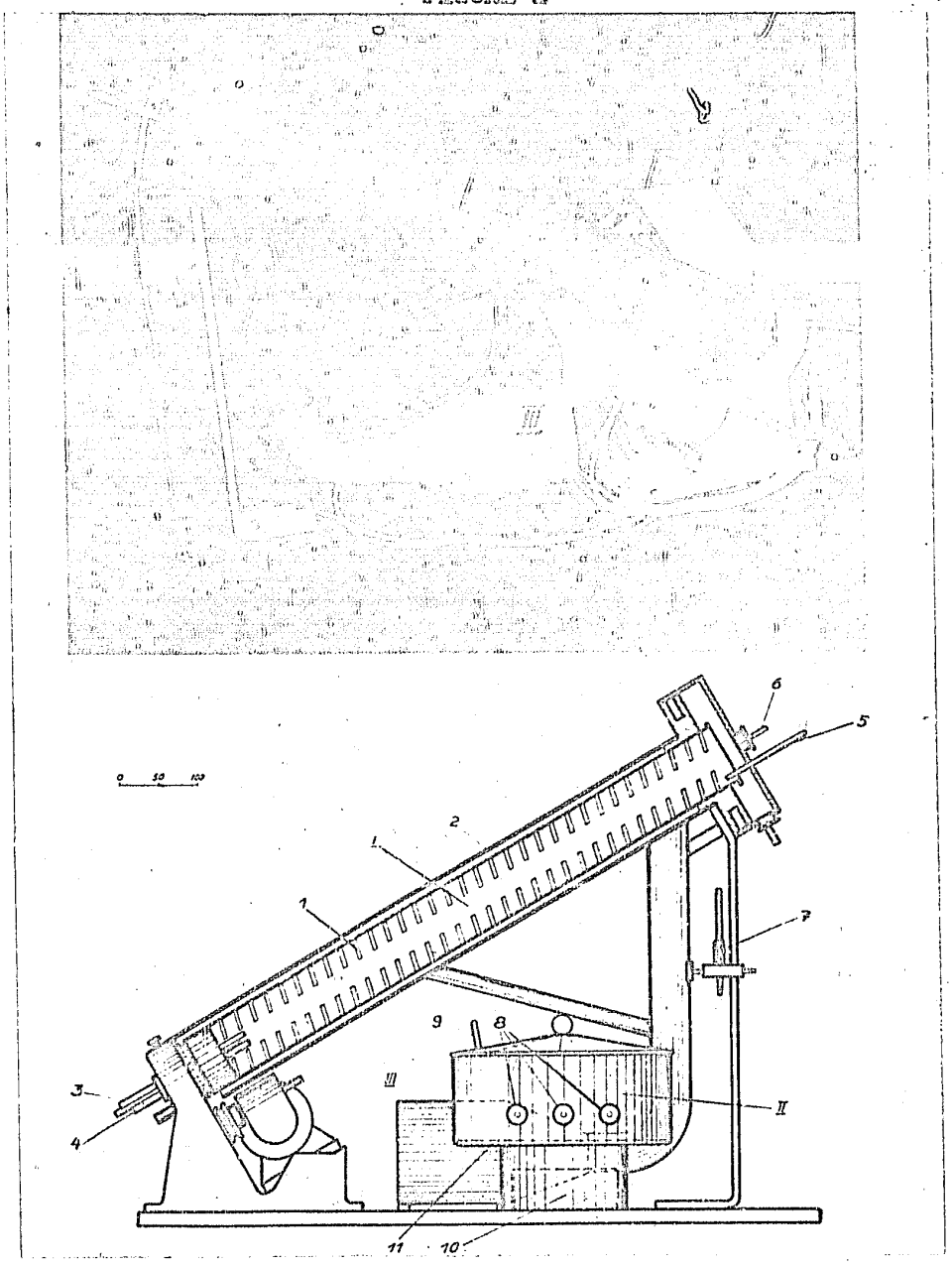
Fetuses were obtained by abdominal hysterotomy which was carried out under general anesthesia. The decision to carry out therapeutic abortion was based on medico-social or social-medical indications according to special permission granted, upon request of the patient, by the Royal Medical Board of Sweden. The operation was carried out when the fetuses were of gestational age ranging from sixteen to twenty weeks. Only fetuses that were grossly normal were used in this study.

TECHNIQUE OF PERFUSION

Perfusion of the fetus was carried out by personnel in Dr. Diczfalusy's laboratory using the technique of Westin (68) with only slight modifications. The perfusion apparatus (Figure 1) consisted of an oxygenator (I), perfusion chamber (II) and a heater (III). The perfusion chamber enclosed the fetus completely and ensured a constant temperature and blood volume. The oxygenator served the double purpose of oxygenating the blood for perfusion and of elevating it to a suitable level to create adequate hydrostatic pressure during perfusion of the fetus.

The perfusion chamber was a glass container filled with equal volumes of isotonic saline and 5.5 per cent glucose in which the fetuses were immersed. The container was sealed with its cover and

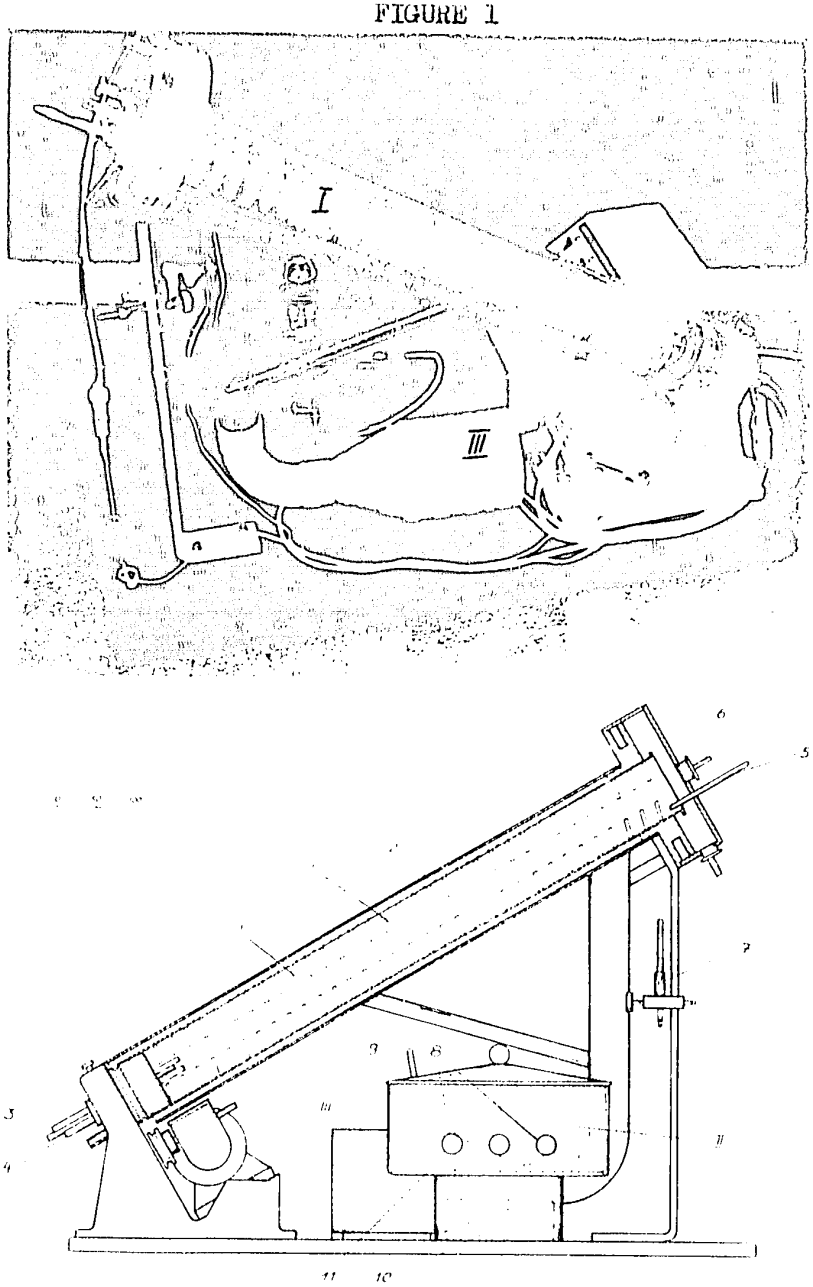
FIGURE 1.



THE PERFUSION APPARATUS

I Oxygenator	1. Spiral	7. O_2 flowmeter
II Perfusion Chamber	2. Cylinder	8. Inlets
III Thermo-controlled heating equipment for oxygenator	3. Inlet	9. Thermometer
	4. Inlet	10. Heater
	5. Thermometer	11. Disc
	6. O_2 inflow	

FIGURE 1



THE PERFUSION APPARATUS

I Oxygenator
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1. Spiral
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6. O_2 inflow
7. O_2 flowmeter
8. Inlets
9. Thermometer
10. Heater
11. Disc

the umbilical catheters were brought out by the side outlets[8]. The temperature of the bath surrounding the fetus was controlled by the regulating device[10] in the narrow part of the chamber beneath the perforated disc[11]. With the chamber filled with fluid and all the air evacuated, the volume of the fetus could not change appreciably and umbilical arterial outflow must therefore equal umbilical venous inflow.

The oxygenator consisted of a rotating cylinder[2] with a spiral[1] attached to its inner wall. The cylinder was placed at an angle of 30° to the horizontal plane and it rotated with a speed of 38 revolutions per minute. Blood introduced at the lower end[3,4] comes either from the fetus or the reservoir of adult human type O, heparinized blood. It was transported to the higher end by the rotating cylinder with its spiral. The blood was oxygenated as it was spread as a thin film on the spiral. The oxygenation was achieved by passing humidified oxygen[6] at 4 litres per minute[7] down the cylinder. Thus with gas exchange, the oxygen tension of the blood was gradually increased and the carbon dioxide tension decreased as the blood was transported to the upper end of the oxygenator. The final oxygen saturation was over 95 per cent. The oxygenated, heparinized blood was collected, filtered to remove foam, and then passed by hydrostatic pressure, into the fetus via the umbilical vein catheter. The blood flow was recorded by a photocell drip counter and the rate was controlled by the hydrostatic pressure. This was usually about 40 cm of water and this could be

altered by changing the height of the perfusion chamber relative to the outflow from the oxygenator. The optimal volume for the entire perfusion system including the fetus was about 250 ml. The system was primed with fresh heparinized, type O, Rh negative adult human blood diluted with 15% saline. The oxygenator was surrounded by a heating jacket and the experiments were carried out at 20°C initially and later at 32°C.

No pre-operative selection of cases was made other than the elimination of recognized abnormal fetuses. The time of day of operation, the induction and maintenance of anesthesia, and the precise operative technique varied from case to case. In all cases, the fetus was obtained by abdominal hysterotomy and the cord was clamped as soon as possible. The fetus was removed from the uterus and placed in a bath of crushed ice and saline. About 3-5 cm. from the umbilicus, small incisions were made into the umbilical vessels and soft plastic siliconized catheters were inserted into the umbilical arteries and vein. The tip of the venous catheter reached the caudal end of the ductus venosus while the tips of the arterial catheters reached just inside the abdominal wall. The positions of the catheters were secured by means of a ligature around the cord.

In random cases bilateral adrenalectomy was carried out following successful insertion of the catheters. This was performed by Dr. R. Wilson using bilateral flank incisions with resection of the twelfth rib on either side. The total operating time was twenty five minutes. Completeness of adrenalectomy was assessed by careful

examination of the operative site at the time of dissection of the organs. In the fetuses undergoing total bilateral adrenalectomy, hydrocortisone was added to the perfusing fluid in a concentration of 23 µg./ml.

Following insertion of the catheters and adrenalectomy the fetus was placed in the perfusion chamber filled with saline. It was maintained at 20°C for the initial studies and at 32°C for the later ones. Blood returning from the fetus via the umbilical arteries was collected in beakers and was not returned to the oxygenator for recycling to the fetus. The inflow drop rate was adjusted to equal the outflow rate. Commencing at time zero and after every fifth minute of perfusion, one-tenth of the radioactive substrate was injected into the umbilical vein catheter. The injection was given slowly over one minute with continuous visual monitoring of the fetus. The final portion of the radioactive substrate was injected in the 45th minute of the perfusion and the perfusion was terminated at 60 minutes. The fetus was removed from the chamber and the chest and abdominal cavities opened. Propylene glycol was the solvent used in dissolving the labelled 4-^{14}C-progesterone. It tends to cause hemorrhage but intrapleural bleeding was found in only one fetus.

POOLING OF TISSUES

At the end of the perfusion the organs were carefully dissected out, blotted free of any blood and weighed. The blood returning from

the fetuses was collected in ethanol and the volume measured.

The tissues from nine grossly normal fetuses were used for the isolation of radioactive metabolites of progesterone. Four fetuses were intact and five were subjected to total adrenalectomy prior to the perfusion. Males and females were processed together but the extracts of the tissues from the intact fetuses were processed separately from those of the adrenalectomized fetuses. Using the two experimental groups, pools were made of the same tissue from each fetus. The sex of the fetus, the crown-heel length in centimeters and the dose of 4-^{14}C-progesterone (in cpm and µg.) perfused is given in Table III.

EXTRACTION OF TISSUES

All tissues were homogenized in 100 per cent ethanol using either the Waring blendor or quartz sand with a mortar and pestle. The homogenate was extracted twice with equal volumes of absolute ethanol and then with three volumes of 80 per cent ethanol. The ethanol extract was taken to dryness on the rotating flash evaporator at 40°C and the residue was then taken up in 70 per cent methanol. This was stored at -20°C for twenty-four hours and then centrifuged at -15°C at 2000 r.p.m. for twenty minutes. This procedure served to defat the extract. The supernatant was collected and reduced to a small volume in vacuo. The pH of this solution was adjusted to 7.2-7.5 with ammonium hydroxide and then it was stored in a deep freeze at -20°C until processed further.

TABLE III

PERFUSION OF PREVIABLE HUMAN FETUSES WITH 4-^{14}C-PROGESTERONE

Sex	Crown-Heel Length (cm)	4-^{14}C-Progesterone cpm x 10^6	Perfused µg.
	INTACT		
M	15.0	21.9	160
M	26.0	24.1	175
F	22.0	24.1	175
F	23.0	25.8	190
	ADRENALECTOMIZED		
M	17.5	23.0	170
M	19.5	23.5	172
M	22.5	26.6	195
F	21.0	28.4	210
F	24.5	23.0	170

PARTITION OF TISSUE EXTRACTS

The slightly alkaline methanolic extract of each tissue pool was dried *in vacuo*, then the residue was taken up in methanol and an aliquot was taken for counting. The remaining extract was taken to dryness again and the residue dissolved in water previously equilibrated with an equal volume of ether. The aqueous solution was transferred to a 500 ml separatory funnel with washings, using a total volume of 100 ml of water, and 100 ml of ether was added to the funnel. The aqueous solution was extracted with the ether by swirling the solutions. This was done carefully to avoid extensive emulsion formation. Stubborn emulsions were cleared either by adding excess ether or by filtering through glass wool. After standing, the lower aqueous phase was run off and the upper ether phase was poured into a second 500 ml separatory funnel containing 100 ml of water previously equilibrated with an equal volume of ether and the extraction repeated. A total of five 100 ml ether strippings were carried through the three one hundred ml water fractions. The water and ether portions were pooled separately and taken to dryness on the flash evaporator. Each extract was dissolved in methanol, an aliquot was taken for counting and the remainder stored in the refrigerator at 4°C until it was further processed. In this manner the metabolites were separated into an ether soluble unconjugated fraction and an aqueous soluble conjugated fraction.

CHROMATOGRAPHY

Column Chromatography. Silica gel chromatography was used for the preliminary separation of the unconjugated (ether soluble) radioactive metabolites. A typical silica gel column was prepared as follows. To twenty-five grams of silica gel (Davidson 100-120 mesh) contained in a beaker, were added ten ml of absolute ethanol with vigorous stirring. Approximately 100 ml of the eluting phase (consisting of 0.25 per cent ethanol in methylene chloride:ligroin B, 1:9) was then added to the silica gel with stirring. A loose fitting plug of cotton wool was placed at the lower end of an all glass column (75 cm long, 1.3 cm O.D.) and the column was filled with the eluting phase. With the stopcock open, the silica gel slurry was poured into the column and the column was tapped with a rubber hammer in order to expel air bubbles and to achieve uniform packing. The dried ether extract of each tissue was dissolved in the eluting phase and transferred to the column with several washings. Five ml fractions were collected using a fraction collector with the rate of flow being twelve drops per minute (15 ml per hour). This column had an elution volume of about 35 ml when checked with Sudan III (69).

A 0.1 ml aliquot was taken from each fraction and the radioactivity was counted and represented graphically by plotting the fraction number on the abscissa against the counts per minute (cpm) in each fraction on the ordinate. The eluting solvent was altered periodically, the changes being governed by the pattern of radioactivity

eluted from the column. The changes in the elution solvent are shown in Table IV. The discrete peaks of radioactivity eluted from the column were pooled, weighed, and the residues stored at 4°C prior to purification by paper chromatography.

Partition column chromatography was used for the separation of the conjugated (water soluble) radioactive metabolites of progesterone. Celite was the column support and it was prepared at room temperature as follows. Equal volumes of Celite (Johns Manville 545) and 0.6 N HCl were mixed and left for twenty-four hours. The Celite was then washed continuously with tap water until approximately neutral. This required about thirty-six hours. The Celite was then washed with four volumes of distilled water, eight volumes of methanol and then spread thinly on a flat tray in the fume hood to dry. The air dried Celite was stored in sealed bottles until used.

A total of 80 to 100 gms of Celite was used for each column with the precise amount of support being chosen in order to obtain a ratio of the weight of sample to weight of support in the range of 1/500 to 1/1000. The solvent system used for the partition column was a slight modification of the system described by Siiteri et al. (70).

Table V outlines the solvent changes that were made as discrete peaks of radioactivity were eluted from the column.

An appropriate amount of Celite was placed in a Waring blendor and it was covered with mobile phase. A volume of stationary phase

TABLE IV

SOLVENT CHANGES ON SILICA GEL COLUMNS

Number of Solvent Change	% Ethanol in Methylene Chloride:Ligroin B
1	0.25 1:9
2	0.5 1:4
3	1.0 1:1
4	1.0 in Methylene Chloride
5	2.0 " "
6	3.0 " "
7	4.0 " "
8	5.0 " "
9	6.0 " "
10	8.0 " "
11	10.0 " "
12	100.0

TABLE V

SOLVENT CHANGES ON CELITE PARTITION COLUMNS

Iso-Octane:t-Butanol/Ammonium Hydroxide:Water	
Initial	300:500/ 25:475
1st change	200:500/ 25:475
2nd change	150:500/ 25:475

equal to one half the weight (in grams) of Celite was slowly added with constant stirring. A cotton wool plug was loosely placed at the lower end of an all glass column (95 cm long, 2 cm O.D.). The column was filled with mobile phase and then the Celite was added to the column and packed tightly and uniformly with a stainless steel Martin Packer (71). The mobile phase was allowed to percolate through the packed column for at least twelve hours before applying the sample. The aqueous fraction was taken to dryness in a 100 ml beaker and six grams of Celite were added. A slurry was made with mobile phase and then 3 ml of stationary phase were added with constant stirring. This mixture was then placed on the column and the top of the column was covered with washed sea sand.

Pressure, in the form of air, filtered through cotton wool and a column of calcium chloride, was applied to the column to obtain a flow rate of approximately twelve drops per minute and 10 ml fractions were collected. A 0.2 ml aliquot was taken from each fraction and was counted. The fractions containing discrete peaks of radioactivity were combined, dried, aliquots removed for counting and stored at $4^{\circ}C$ prior to further chromatographic purification.

Paper Chromatography. Strips of unwashed Whatman No. I filter paper, 17 X 56 cm were used. The solvent systems used are outlined in Table VI. In the Bush type systems the papers were first equilibrated in an atmosphere of mobile and stationary phase for at least six hours, and then the chromatogram was developed with mobile phase. In the Zaffaroni type systems the papers were impregnated with

TABLE VI

PAPER PARTITION CHROMATOGRAPHY

System	Solvent	Reference	Type
Unconjugated Steroids			
A	Ligroin C/Propylene Glycol	72	ZAFFARONI
B	Ligroine (1)/90% Methanol (1)	73	BUSH
C	Benzene:Cyclohexane (1:1)/Propylene Glycol	74	ZAFFARONI
D	Toluene/Propylene Glycol	75	ZAFFARONI
E	Benzene (500)/Methanol (250):Water (250)	76	BUSH
F	Iso-octane (400)/t-Butanol (360):Water (120)	77	BUSH
G	Iso-octane (100):Toluene (300)/Methanol (400):Water (100)	78	BUSH
Conjugated Steroids			
H	Iso-propyl ether (300):t-Butanol (200)/ Ammonium Hydroxide (50):Water (450)	79	BUSH
J	n-Butyl-ether (300):t-Butanol (200)/ Ammonium Hydroxide (20):Water (180)	79	BUSH
K	Toluene (250):n-Butanol (250)/ Ammonium Hydroxide (25:Water (450)	79	BUSH

propylene glycol:methanol (1:1) and blotted just prior to application of the samples. These papers were not equilibrated with the solvents prior to development of the chromatograms. All chromatography was carried out at room temperature.

When the Δ^4-3-keto steroids were used as standards, they were located on the chromatograms by means of ultra-violet light source (Chromatovue 254 mμ maximum). The 17-ketosteroid standards were located on paper by the use of the Zimmerman reaction as described by Savard (58). The paper strip was dipped in a solution of 2.5 N potassium hydroxide in absolute ethanol, blotted dry, and then dipped in 2 per cent meta-dinitrobenzene in absolute ethanol. The 17-ketosteroids appeared as violet-blue spots after heating to 60-70°C. Ring A reduced steroids were detected by dipping the paper strip into a solution of 10 per cent phosphomolybdic acid in ethanol, blotting the strip, and heating in an oven to 60-70°C (80). The steroids appeared as dark blue-green spots. Radioactive metabolites were located on paper chromatograms by scanning a linear strip, 3 cm wide, cut from the middle of the developed chromatogram in a windowless gas flow 4π scanner (Vanguard Instruments Model 800).

Metabolites were eluted from the paper chromatogram by hanging the selected area of paper on the end of a curved stainless steel needle which was attached to a 10 ml. all glass syringe. The solvent used for elution ran down over the paper and was collected as drops from the end of the paper. Two 10 ml portions of methanol were used for the elution of each area of paper containing labelled steroid.

The eluate from the Zaffaroni type systems usually contained some propylene glycol which increased the weight of the extract obtained. For this reason the residue of the material eluted was subjected to an ethyl acetate:water partition. The unconjugated steroids were partitioned into the ethyl acetate layer which was then dried over anhydrous sodium sulphate and evaporated *in vacuo*. Such residues were then ready for the next chromatogram or for crystallization.

DETERMINATION OF RADIOACTIVITY

Aliquots of samples to be counted were evaporated under nitrogen in 5 dram vials (Wheaton Glass Company) and the residue was dissolved in 5 ml toluene containing 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-2(5-phenyloxazolyl)-benzene. Compounds which were insoluble in toluene such as very polar unconjugated steroids, or the conjugated steroids were dissolved in 0.1 ml or 0.2 ml methanol prior to the addition of the toluene scintillation mixture. To correct for quenching a ^{14}C internal standard dissolved in 0.3 ml of toluene was added to each vial which was then recounted.

A Packard Tri-Carb liquid scintillation Spectrophotometer (Model 3214) was used for counting. All the experiments to be described involved the use of only one isotope, ^{14}C. The counting efficiency for ^{14}C was 90% with the photomultiplier set at a gain of 60% and with the lower discriminator set at 50 and the upper discriminator set at infinity. This efficiency was determined using standard ^{14}C

hexadecane (CFR Batch 5, Amersham, England). The background counting rate at these settings was between 33 and 36 cpm. In all instances sufficient counts were accumulated to give a standard error of no more than 2%.

PURITY OF SUBSTRATE

4-^{14}C-progesterone (specific activity 69 μc/mg) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England (CFA-148, Batch 17). The labelled steroid was purified by thin layer chromatography on silica gel G using system H of Lisbon (61). It was stored at 4°C in benzene:methanol (3:2). Between 160 and 200 μg of progesterone were used in each of the perfusion experiments. The labelled progesterone was dissolved in 1.4 ml of propylene glycol and one-tenth of the total volume was injected into the umbilical vein catheter every fifth minute commencing at time zero and finishing in the forty-fifth minute.

FORMATION OF DERIVATIVES

All derivatives were formed on a milligram scale using the mixture of the weightless radioactive metabolite and the non-radioactive carrier.

1. Acetates

The acetates were prepared by dissolving the steroid in pyridine and adding acetic anhydride in a ratio of 2:1. The minimum volumes of reagents used were 0.2 ml of pyridine and 0.1 ml of acetic

anhydride. After the mixture was left at room temperature for eighteen hours, the reaction was terminated by transferring the solution to a 250 ml separatory funnel containing 50 ml of ice water and 1 ml of 6 N sulphuric acid. This solution was then extracted three times with 100 ml of ethyl acetate and the combined ethyl acetate extracts were washed with 6 N sulphuric acid (3 x 10 ml), 1 N sodium hydroxide (3 x 10 ml or until basic) and with distilled water (3 x 10 ml or until neutral). The ethyl acetate was then dried over anhydrous sodium sulphate, filtered, and evaporated. The acetates thus formed were then chromatographed on small alumina columns prior to crystallization. The solvent systems used on these small columns varied, depending on the acetate formed.

2. Reduction

Sodium borohydride reduction was performed as described by Norymberski and Wood (82). The product of the reaction was then chromatographed on a small alumina column.

3. Chromic Acid Oxidation

The oxidative cleavage of the side chain at position C-17 was carried out using the method described by Solomon et al. (83). Acetic acid was purified each time by refluxing it for four hours over potassium permanganate (3 per cent w/v), and then it was distilled. The fraction that came off at 117-118°C was collected. A 90 per cent acetic acid solution in water was made up and this in

turn was used to make a two per cent chromic acid solution (w/v). Between 12 and 15 mg of steroid were dissolved in 0.5 ml of the acetic acid and 0.3 ml of the two per cent chromic acid solution was added. A control tube containing all the solutions but no steroid was prepared. The original solution was a dark colour and changed to a green-brown colour. When the green colour appeared a second 0.3 ml of chromic acid was added and the reaction allowed to proceed for an additional two hours. The reaction was stopped by the addition of 25 ml of water. The aqueous reaction mixture was extracted with ethyl acetate (3 x 50 ml) and the pooled ethyl acetate extracts were washed with 1 N sodium hydroxide (3 x 10 ml or until basic) and distilled water (3 x 10 ml or until neutral). The ethyl acetate was dried over anhydrous sodium sulphate, filtered, and evaporated on the flash evaporator and the residue was then chromatographed on an alumina column.

SOLVOLYSIS

Steroid sulphates were cleaved by solvolysis, using a slight modification of the method described by Burstein and Lieberman (84). A total of 100 ml of freshly refluxed and distilled tetrahydrofuran was equilibrated with 100 ml of distilled water at pH 1. Sodium chloride was added to the water to give a 20 per cent solution (w/v). Then 50 ml of the equilibrated tetrahydrofuran was added to the dry steroid residue and 70 per cent perchloric acid was added to the mixture (0.09 ml per 100 ml of tetrahydrofuran). The mixture was

incubated for 18 hours at 37°C and then it was neutralized by the addition of 5 ml of concentrated ammonium hydroxide per litre of solution. The solution was reduced in volume and then diluted to 25 ml with distilled water. This solution was then extracted with ethyl acetate (3 x 25 ml) and the pooled ethyl acetate extract was washed with 1 N sodium hydroxide (3 x 5 ml or until basic) and then with distilled water (3 x 5 ml or until neutral). The ethyl acetate was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness.

β-GLUCURONIDASE HYDROLYSIS

The aqueous solution to be hydrolyzed with β-glucuronidase was adjusted to pH 4.7 using 50 per cent sulphuric acid (v/v) or 1 N sodium hydroxide. To the aqueous solution was then added 2 per cent by volume of 2 Molar acetate buffer, pH 4.7. To the solution was added β-glucuronidase at a concentration of 600 units per ml. Then 5 ml of methylene chloride was added to the mixture to control bacterial growth, and the mixture was incubated at 37°C for five days. Then the liberated steroid was extracted with ethyl acetate (3 x 40 ml) and the pooled ethyl acetate extracts were washed with 0.1 N sodium hydroxide (3 x 10 ml or until basic) and with distilled water (3 x 10 ml or until neutral). The neutral extract was dried over anhydrous sodium sulphate, filtered, and evaporated to dryness to give a residue which was purified by chromatography.

COLUMN CHROMATOGRAPHY PRIOR TO CRYSTALLIZATION

To facilitate crystallization following reduction, acetylation and other reactions the product formed was purified by chromatography. This sometimes involved paper chromatography as discussed earlier or more commonly the use of small columns in which the supporting medium was either silica gel or alumina.

The silica gel (Davidson, 100-120 mesh) was used as purchased, in amounts ranging between 50 and 100 times the weight of the steroid being purified.

Harshaw alumina (200 mesh) was deactivated by the procedure previously described by Solomon et al. (82). The columns were packed as described earlier and were developed with variable ratios of ligroin B to benzene.

RADIOCHEMICAL PURITY

Tentative identification of the various metabolites isolated from the tissue extracts was achieved by comparing the mobility of the unknowns with selected authentic standards in different paper chromatographic systems. When the mobility of the labelled metabolite corresponded to a known standard, the essentially weightless radioactive materials were eluted from the paper chromatograms and were mixed with an appropriate weight of pure non-radioactive carrier. Knowing the amount of radioactivity added (cpm) plus the weight (mg) of carrier, it was possible to calculate the expected specific activity (cpm/mg). The mixture

was then crystallized two to four times using appropriate solvent pairs. Following each crystallization the mother liquor was carefully aspirated from the crystals. The crystals were washed twice with a suitable non-polar solvent and the washings added to the mother liquor. The crystals and mother liquors were carefully dried under nitrogen. An aliquot of the crystals, corresponding to 0.5 to 1.0 mg was removed and accurately weighed on a Mettler microbalance. They were quantitatively transferred to a counting vial, dissolved in methanol, dried under nitrogen, and then the radioactivity counted in toluene phosphor. The mother liquor was quantitatively transferred to a volumetric flask. An aliquot was transferred to a vial, and counted.

When the specific activities of the crystals and mother liquor were within the limits of experimental error after two or more crystallizations a suitable derivative was made and it was purified by column chromatography. The derivative was then recrystallized two to four times. The metabolite was accepted as being radiochemically pure when the specific activity of two successive crystals and mother liquors differed by less than 10 per cent. Infra-red spectra of each derivative formed were obtained on a Perkin Elmer model 221 spectrophotometer. The spectra were obtained using a solution in carbon disulphide or as KBr disks.

PREPARATION OF STEROID SULPHATES

Sulphates were prepared using a method similar to that described by Fieser (85). The unconjugated steroid was dissolved in 1.0 ml of dry pyridine and this solution was transferred to a tube immersed in ice, containing a freshly prepared mixture of 1.0 ml pyridine and 0.2 ml of chlorosulphonic acid. After standing in ice for twenty minutes, twenty volumes of ether were added to precipitate the pyridinium sulphate. The sulphate solution was centrifuged, the supernatant removed and the residue washed twice with ether. The resulting residue was dissolved in 30 ml of 1 N ammonium hydroxide and this solution was extracted with n-butanol using 15, 10, and 5 ml portions of solvent. The n-butanol was removed in vacuo and the residue was dissolved in methanol, filtered, and crystallized. The melting points and infrared spectra of the products were compared to those of authentic standards when possible. Sulphates were detected on paper chromatograms using the method described by Roy (86). The paper strip was dipped in a solution of chloroform:methylene blue (1:5). The sulphate area turned blue.

SOLVENTS AND REAGENTS

All solvents were of reagent grade and were distilled prior to use.

RESULTS

The experimental results to be described were obtained following perfusion experiments carried out in four intact and five totally adrenalectomized previable human fetuses. Purified 4-^{14}C-progesterone dissolved in propylene glycol was injected into the umbilical vein perfusion catheter every fifth minute in ten equal amounts. Oxygenated and heparinized type O adult human blood was perfused in single cycle throughout the entire one hour experiment. At the end of the perfusion all the tissues were extracted and defatted. The same tissues within each experimental group were pooled to make one from the intact and the other from the adrenalectomized fetuses. Then the metabolites were separated and partially identified by column and paper chromatography and the final identification and degree of radiochemical purity was established by crystallization following addition of unlabelled carrier steroid, derivative formation and recrystallization.

GENERAL

The sex, crown-heel length and total amount of radioactivity perfused in the four intact and five adrenalectomized fetuses are shown in Table III. The gestational age of the fetus at the time of interruption of the pregnancy was calculated from the first day of the last normal menstrual period and was between 16 and 20 weeks.

In Table VII are shown the total blood volumes and tissue

TABLE VII

RADIOACTIVITY IN TISSUE POOLS FROM FOUR INTACT FETUSES PERFUSED WITH 4-^{14}C-PROGESTERONE

Tissue	Volume (ml)	Tissue Extract cpm x 10^6	Ether Phase cpm x 10^6	Ether Phase % of Total	Aqueous Phase cpm x 10^6	Aqueous Phase % of Total
Perfusate I (0 - 15 min)	152	5.59	5.27	94.3	0.19	3.4
Perfusate II (16 - 30 min)	163	8.21	7.83	95.5	0.45	5.5
Perfusate III (31 - 45 min)	166	9.38	7.76	82.7	0.81	8.6
Perfusate IV (46 - end)	180	6.36	5.88	92.5	0.56	8.8
	Weight (gm)					
Adrenal	3.6	0.57	0.31	54.2	0.22	38.6
Liver	38.9	5.81	4.97	85.7	0.61	10.5
Lung	34.1	1.78	1.57	88.2	0.12	6.8
Kidney	7.6	0.39	0.34	87.0	0.05	12.8
Intestine	26.5	1.23	1.07	86.2	0.17	13.9
Residue	738.0	6.89	7.99	116.0	0.90	13.1

weights from each pool of tissue following the perfusion experiment in four intact fetuses. Also shown are the total cpm in each tissue pool. Due to an oversight these values were not corrected for quenching, thus accounting for the apparent discrepancies in the counts shown in this table (e.g. residue). The cpm and per cent of total counts in the ether fraction and aqueous fraction after partitioning are included in this table. Table VIII shows similar data on the distribution of radioactivity in the tissue pools from the five perfusions in totally adrenalectomized fetuses. These counts were corrected for quenching.

THE ISOLATION, IDENTIFICATION AND PROOF OF RADIOCHEMICAL PURITY OF METABOLITES ISOLATED FROM THE FETAL TISSUES

ETHER SOLUBLE METABOLITES ISOLATED FROM THE TISSUES OF INTACT FETUSES

Each tissue extract was chromatographed initially on a silica gel column using a discontinuous gradient. The solvents used to develop this column were ethanol, methylene chloride and ligroin B. An increasing concentration of ethanol in a changing ratio of methylene chloride:ligroin B was used to elute the metabolites. An aliquot of each column fraction was counted and the radioactivity eluted served as a guide in changing the solvents applied to the column. This type of chromatography afforded a good initial separation of the metabolites of progesterone in each tissue studied.

TABLE VIII

RADIOACTIVITY IN TISSUE POOLS FROM FIVE ADRENALECTOMIZED FETUSES PERFUSED WITH 4-^{14}C-PROGESTERONE

Tissue	Volume (ml)	Tissue Extract cpm x 10^6	Ether Phase cpm x 10^6	Ether Phase % of Total	Aqueous Phase cpm x 10^6	Aqueous Phase % of Total
Perfusate I (0 - 15 min)	292	6.68	7.43	111.0	0.26	3.9
Perfusate II (16 - 30 min)	385	9.63	9.60	99.7	0.47	4.9
Perfusate III (31 - 45 min)	330	7.21	8.77	122.0	0.36	5.0
Perfusate IV (46 - end)	384	7.10	7.29	103.0	0.24	3.4
	Weight (gm)					
Liver	44.9	6.37	6.48	102.0	0.34	5.3
Lung	35.1	1.39	1.62	117.0	0.07	5.0
Kidney	7.8	0.29	0.30	103.0	0.02	6.9
Intestine	31.4	1.18	1.11	94.0	0.13	11.0
Residue	850.0	12.84	18.27	142.0	0.59	4.6

The fractions containing discrete peaks of radioactivity eluted were pooled, dried, weighed and aliquots were counted. Sequential paper chromatography was then carried out using the systems outlined in Table VI.

Crystallization of the radioactive metabolite with authentic carrier, derivative formation and recrystallization were carried out using the techniques discussed previously. On certain occasions following initial crystallization the same compounds isolated from different tissues were pooled for derivative formation and re-crystallization.

ADRENALS

The separation of radioactive metabolites following silica gel column chromatography of the ether soluble material from the adrenals is shown in Figure 2. The fractions pooled and any compounds identified from the pool are shown in Table IX.

Paper partition chromatography of the individual radioactive peaks shown in Table IX was carried out. Some of the labelled material from the column resolved into a number of small zones after paper chromatography and identification of these was precluded because they did not contain a sufficient amount of radioactivity. Table X shows the paper chromatographic systems used in the purification of the identified metabolites of the adrenal ether fraction. Also shown is the radioactivity expressed as cpm eluted from the final paper plus the weight in mg of the carrier steroid added prior to

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE ADRENAL FROM THE INTACT FETUS

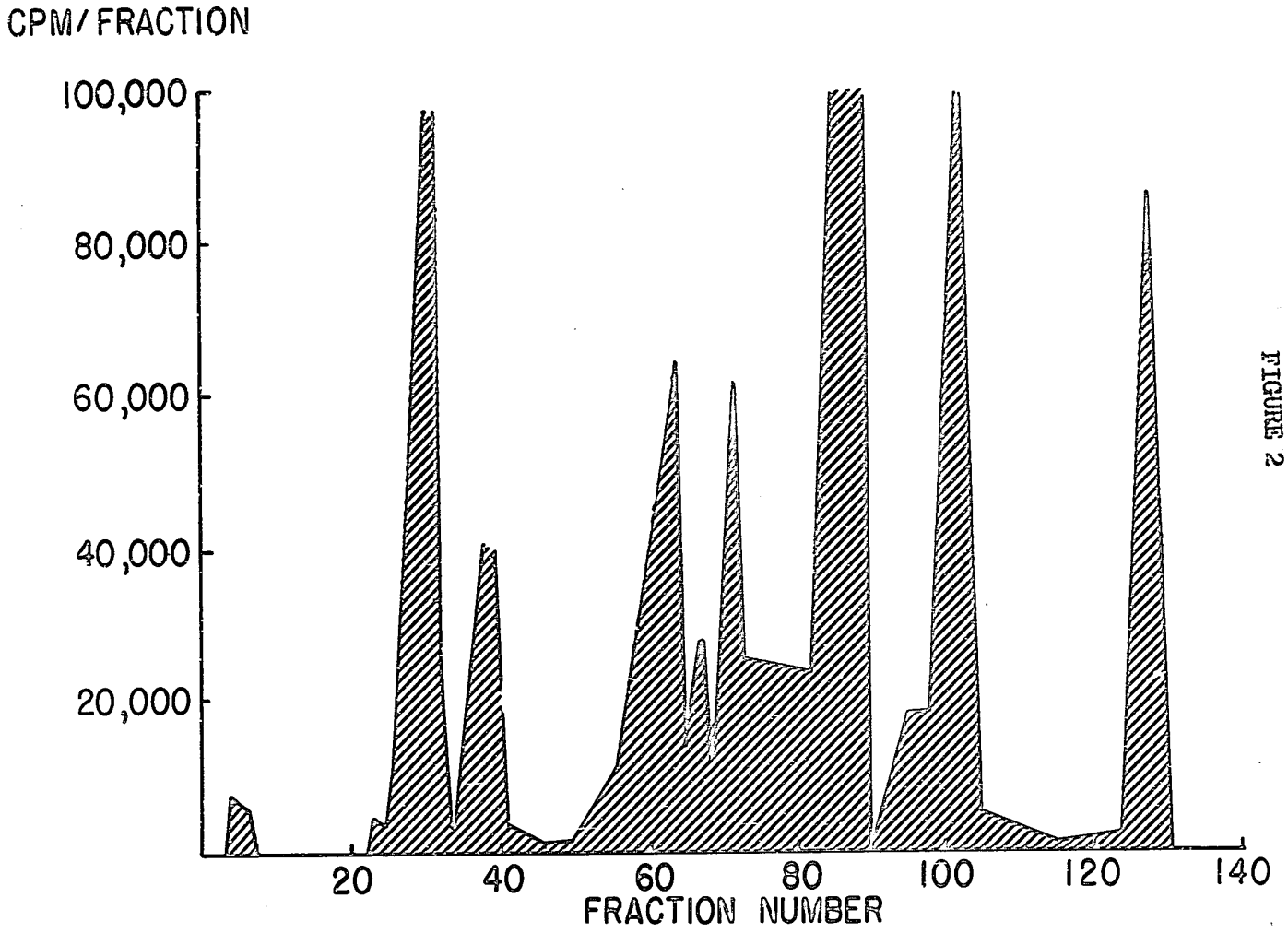

FIGURE 2

TABLE IX

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE ADRENAL

Fractions	cpm	Metabolite Identified
28-34	36,000	Progesterone
35-42	17,600	Two unknowns
55-67	35,500	20α-dihydroprogesterone 17α-hydroxyprogesterone
68-76	23,800	Two unknowns
77-82	17,000	One unknown
83-89	66,200	16α-hydroxyprogesterone
90-97	17,300	One unknown
98-106	36,800	One unknown
107-125	7,900	Two unknowns
126-129	4,300	Two unknowns

TABLE X

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE ADRENAL FROM THE PERFUSED INTACT FETUS

Compound	20α-dihydroprogesterone	17α-hydroxyprogesterone	16α-hydroxyprogesterone
Cpm eluted from silica gel column	35,500		66,200
Paper chromatography systems used to purify metabolites	C → A	C → A	D → C
Cpm eluted from final paper	9,300	4,500	15,000
Carrier steroid (mg) added to eluate	15.80	23.60	27.00

crystallization. Three metabolites were identified from the adrenal ether fraction. These are 20α-dihydroprogesterone, 16α-hydroxy-progesterone and 17α-hydroxyprogesterone. The crystallization data of the steroid and its appropriate derivative is shown in Table XI. The side chain of 17α-hydroxyprogesterone was oxidized with chromic acid to yield androstenedione. It should be noted that the pure 20α-dihydroprogesterone obtained by crystallization was then mixed with the same purified metabolite isolated from perfusate and liver. This mixture was then acetylated and crystallized. This accounts for the widely different specific activity of the free compound and its derivatives.

PERFUSED BLOOD

The perfusate returning from the fetus via the umbilical artery catheter(s) during the one hour experiment was collected at 15 minute intervals (numbered 1-4). Each portion was processed separately but no consistent differences were noted in comparing the results obtained for the four samples. Illustrated in Figure III is the elution of radioactivity from the silica gel column when the extract of perfusate (number 4) was chromatographed. This figure is representative of the four blood extracts which were processed.

The metabolites in each blood extract were investigated separately. In Tables XII-XV are shown the fractions pooled from each column, the amount of radioactivity in the pool, and the metabolite, if any, ultimately identified in that peak.

TABLE XI

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT FROM THE ADRENAL OF THE PERFUSED INTACT FETUS

(specific activities - cpm/mgm)

	20α-dihydroprogesterone		17α-hydroxyprogesterone		16α-hydroxyprogesterone	
Crystallization	XL	ML	XL	ML	XL	ML
1	660	635	150	300	560	530
2	625	630	145	165	540	580
3	655	615	135	150		
Calculated	590		190		555	

	20α-dihydroprogesterone acetate*		Δ^4-androstenedione		16α-hydroxyprogesterone acetate	
	XL	ML	XL	ML	XL	ML
1	7520	7500	165	170	500	530
2	7480	7400	170	155	500	520
3	7480	7350				
Calculated	8030		160		490	

*The discrepancy in specific activities between 20α-dihydroprogesterone and its acetate is due to the combination of 20α-dihydroprogesterone from liver and perfusate (following crystallization in the free form) with that from the adrenal, then acetylation and recrystallization of this mixture.

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE PERFUSATE FROM THE INTACT FETUS

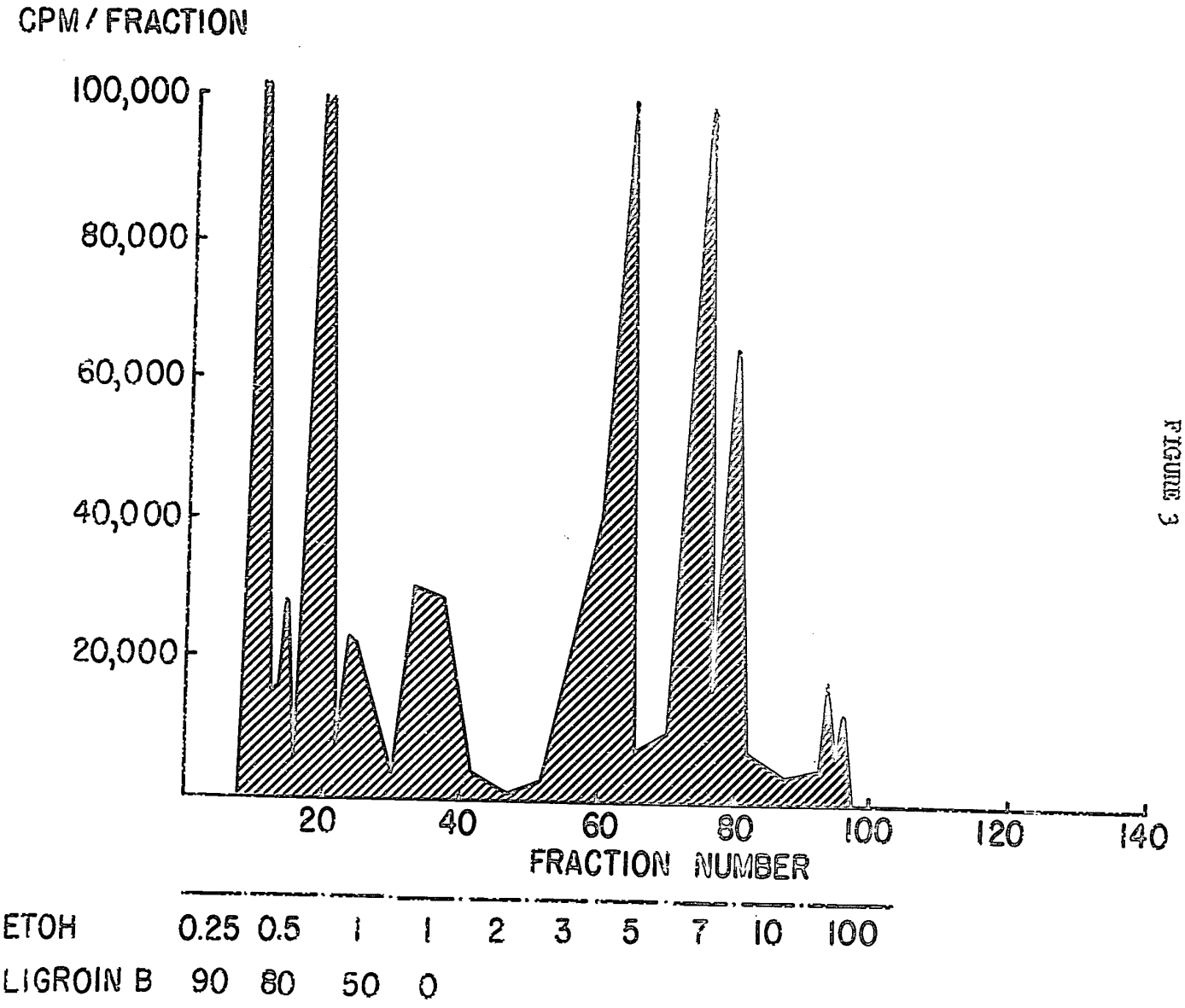

FIGURE 3

TABLE XII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE I

Fractions	cpm	Metabolite Isolated
22-44	4,831,000	Progesterone
52-64	41,000	20α-dihydroprogesterone
65-76	149,000	16α-hydroxyprogesterone
77-86	19,000	Corticosterone
115-117	18,000	Unknown

TABLE XIII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE II

Fractions	cpm	Metabolite Isolated
1-11	175,000	Unknown
12-25	6,475,000	Progesterone
26-36	61,000	Pregnanolone
43-68	101,000	20α-dihydroprogesterone
69-79	63,000	20α-dihydroprogesterone
80-91	240,000	16α-hydroxyprogesterone plus unknown
92-97	38,000	16α-hydroxyprogesterone plus unknown
98-104	22,000	Corticosterone
105-125	51,000	Two unknowns
126-137	36,000	Two unknowns

TABLE XIV

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE III

Fractions	cpm	Metabolite Isolated
3-11	233,000	Unknown
12-22	6,163,000	Progesterone
23-32	116,000	Progesterone, Pregnanolone
33-42	99,000	Progesterone, Pregnanolone
50-64	396,000	20α-dihydroprogesterone
67-73	636,000	16α-hydroxyprogesterone plus unknown
74-95	159,000	16α-hydroxyprogesterone plus unknown
96-100	28,000	Unknown

TABLE XV

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE IV

Fractions	cpm	Metabolite Isolated
6-10	9,000	Unknown
18-30	295,000	Progesterone plus unknown
31-37	4,330,000	Progesterone, Pregnanolone
38-52	198,000	Pregnanolone
57-64	197,000	20α-dihydroprogesterone
65-77	237,000	Three unknowns
78-89	112,000	Corticosterone plus unknown
90-105	35,000	Two unknowns
106-115	37,000	Four unknowns
124-127	26,000	One unknown

The four metabolites isolated from the perfusate were present in the samples collected at the various time intervals and in Table XVI are shown the procedures used to isolate the metabolites. The paper chromatography systems used for each compound are shown along with the number of cpm eluted from the final paper. The amount of authentic carrier in mg mixed with the radioactive metabolite for crystallization is also shown. The crystallization data for the free compounds and their derivatives are shown in Table XVII. Pregnanolone, 20α-dihydroprogesterone, 16α-hydroxy-progesterone and corticosterone were isolated from the perfusate and the radiochemical purity of each was established.

Following initial crystallization of the pregnanolone, the first mother liquor was recrystallized with the epimer 3β-hydroxy-5α-pregnan-20-one. The continued association of radioactivity with the carrier in the crystals plus the formation of the derivative and its satisfactory crystallization is taken as evidence that these epimers are present in the extract of the perfusate.

TABLE XVI

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF PERFUSATES

Metabolite	20α-dihydroprogesterone	Corticosterone	16α-hydroxyprogesterone	Pregnanolone
Perfusate	I	I	II	III
Cpm eluted from silica gel column	41,000	19,000	38,000	99,000
Paper chromatography systems used to purify metabolites	B ↓ C	D ↓ C	B ↓ C	A
Cpm eluted from final paper	12,150	3,900	11,500	38,200
Carrier steroid (mg) added to eluate	14.75	33.55	13.05	31.20

TABLE XVII

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF THE METABOLITES ISOLATED FROM THE ETHER EXTRACTS OF THE PERFUSATES OF THE INTACT FETUS

(specific activities - cpm/mg)

	20α-dihydro-progesterone		Corticosterone		16α-hydroxy-progesterone		Pregnanolone		3β-hydroxy-α-pregnan-20-one	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML	XL	ML
1	640	950	120	180	590	980	340	5130	600	1750
2	640	640	110	120	560	610	300	780	690	820
3	650	670	120	110	570	560	280	340	710	720
4							290	300		
Calculated	820		120		850		220		960	

	20α-dihydro-progesterone Acetate*		Corticosterone Acetate		16α-hydroxy-progesterone Acetate		3α,20β-dihydroxy-5β-pregnane		3β,20β-dihydroxy-5α-pregnane	
	XL	ML	XL	ML	XL	ML	XL	ML	XL	ML
1	4350	4540	90	90	440	420	270	270	740	710
2	4260	4300	90	90	440	440	270	260	720	720
3	4290	3960								
Calculated	4200		110		440		290		710	

*The discrepancy in specific activities between 20α-dihydroprogesterone and its acetate is due to the combination of 20α-dihydroprogesterone from adrenal and liver (following its crystallization) with that from the perfusate, then acetylation and crystallization of this mixture.

LIVER

A twenty-five gram silica gel column developed with a discontinuous gradient using increasing concentrations of ethanol in methylene chloride:ligroin B was used for the preliminary purification of the liver extract from the perfusion studies in the intact fetus. The graphic representation of the eluted radioactivity is shown in Figure 4. The fractions pooled, the total cpm in each pool and the metabolites identified are shown in Table XVIII. Individual radioactive peaks from these pools were subjected to descending paper chromatography. The systems used and the cpm recovered from the final paper for the three compounds identified are shown in Table XIX. As shown in this table, less than half of the isolated radioactivity (551,400 cpm) was mixed with carrier steroid for crystallization. The crystallization data for these compounds are given in Table XX.

LUNG

The initial separation of the radioactive metabolites of progesterone was achieved by absorption column chromatography using silica gel as the supporting medium. The column was developed using serial solvent changes involving increasing concentrations of ethanol in methylene chloride and ligroin B. In Figure 5 is shown the elution of radioactivity, determined by counting an aliquot of each of the 5 ml fractions. Certain fractions from the radioactive peaks

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE LIVER FROM THE INTACT FETUS

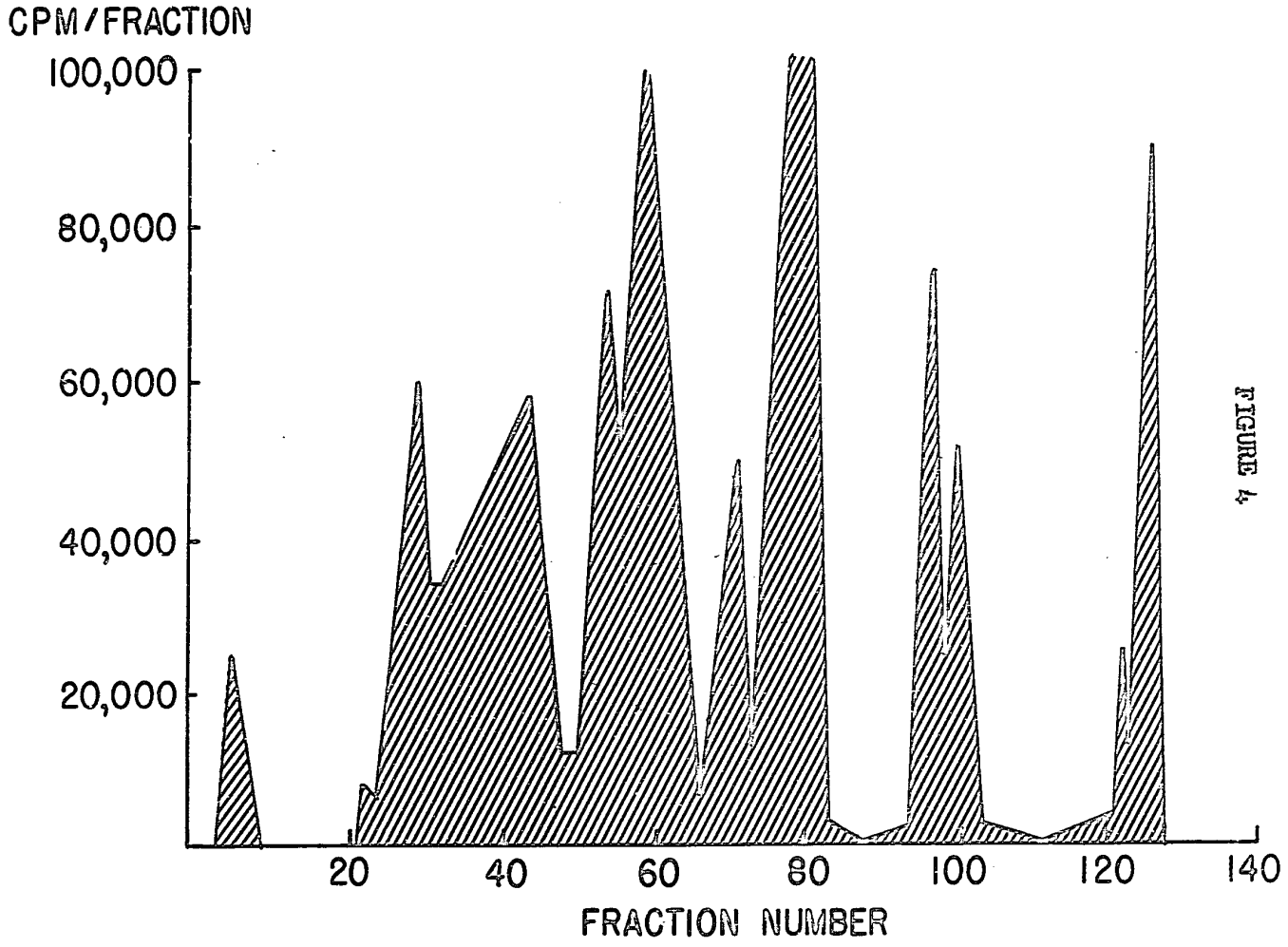

FIGURE 4

TABLE XVIII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE LIVER

Fractions	cpm	Metabolite Isolated
5-12	62,150	Unknown
16-27	22,650	Unknown
28-35	297,600	Progesterone
36-53	503,000	Pregnanolone plus progesterone
54-56	168,400	Pregnanolone plus unknown
57-69	716,600	20α-dihydroprogesterone
70-77	209,050	Three unknowns
78-93	1,664,000	Pregnanediol
94-100	132,500	Two unknowns
101-112	243,700	Two unknowns
123-129	121,200	One unknown

TABLE XIX

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE LIVER

Metabolite	Pregnanolone	20α-dihydroprogesterone	Pregnanediol
Cpm eluted from silica gel column	503,100	716,600	1,665,000
Paper chromatography systems used to purify metabolite	B ↓ A	C	B ↓ C
Cpm eluted from final paper	354,900	503,600	1,220,000
Carrier steroid (mg) added to eluate	100.0	21.65	551,400 cpm 110.92

TABLE XX

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT FROM THE LIVER OF THE PERFUSED INTACT FETUS

(specific activity - cpm/mg)

Crystallization	20α-dihydroprogesterone		Pregnanolone		Pregnanediol	
	XL	ML	XL	ML	XL	ML
1	23340	23100	2570	4970	5350	4310
2	23090	23020	2570	4220	5120	4650
3	23270	23270	2410	3240	5200	5330
4			2560	2900		
Calculated	23260		3350		4980	

	20α-dihydroprogesterone Acetate*		30α,20β-dihydroxy-5β-pregnane		Pregnanediol Diacetate	
	XL	ML	XL	ML	XL	ML
1	7520	7500	5350	4310	4240	4090
2	7480	7400	5120	4650	4380	4330
3	7480	7350	5200	5330	4360	4040
Calculated	7620		4900		4160	

*Following initial crystallization of 20α-dihydroprogesterone isolated from liver and adrenal extracts, the final crystals of each were mixed together, acetylated and recrystallized. This accounts for the difference in specific activities of the acetate compared to the free compound.

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE LUNG FROM THE INTACT FETUS

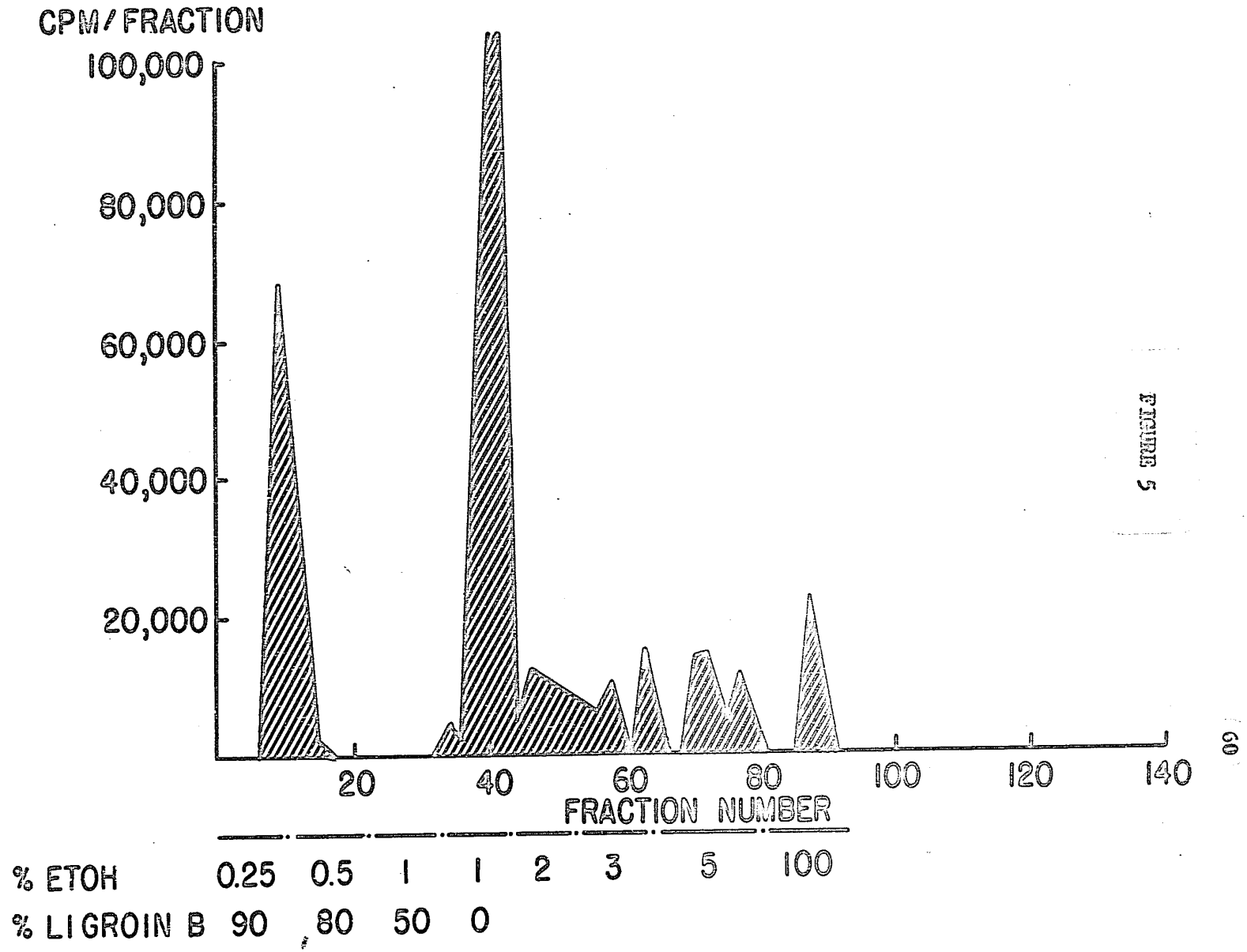

FIGURE 5

were combined as shown in Table XXI. The total cpm in each peak is shown along with any metabolite isolated. Further purification of the radioactive material was achieved by paper chromatography using the specific systems shown in Table XXII. The cpm recovered from the final paper and the amount of authentic carrier steroid added to the labelled metabolite prior to recrystallization are also shown in this table.

The crystallization data of these metabolites are shown in Table XXIII. The detection and proof of identity of the epimers of pregnanolone was first demonstrated at this time. Prior to this only the 3α,5β epimer had been isolated but the mother liquors of its crystallizations had contained large amounts of radioactivity. Included in this table is data from an attempt to crystallize the 5β-form with radioactivity eluted from the paper. Most of the radioactivity appeared in the first mother liquor and very little radioactivity remained with the crystalline material suggesting that there was little if any of the 5β-form. The first mother liquor was then mixed with the 5α-epimer and crystallized with the good results as shown in the table.

KIDNEY

The ether extract from the kidneys obtained from the perfused intact fetuses was subjected to absorption column chromatography on silica gel as the initial purification procedure. Approximately 100 five ml fractions were collected using a discontinuous gradient

TABLE XXI

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE LUNG EXTRACT

Fractions	cpm	Metabolite Isolated
7-13	125,000	Unknown
27-34	30,000	Unknown
35-42	946,000	Progesterone
43-53	107,000	Progesterone, Pregnanolone
54-59	38,000	17α-hydroxyprogesterone
62-66	42,000	20α-dihydroprogesterone
67-76	65,000	6β-hydroxyprogesterone plus unknown
82-86	32,000	Two unknowns

TABLE XXII

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE LUNG

Metabolite	Pregnanolone	17α-hydroxy-progesterone	20α-dihydro-progesterone	6β-hydroxy-progesterone
Cpm eluted from silica gel column	107,000	38,000	42,000	65,000
Paper chromatography systems used to purify metabolite	A	B ↓ C	C	C ↓ A
Cpm eluted from final paper	56,350	900	21,350	16,800
Carrier steroid (mg) added to eluate	45.62	10.95	15.20	17.40

TABLE XXIII

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE LUNG FROM THE PERFUSATE INTACT FETUS

(specific activities - cpm/mg)

	Pregnanolone		3β-hydroxy-5α-pregnan-20-one		17α-hydroxy-progesterone		20α-dihydro-progesterone		6β-hydroxy-progesterone	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML	XL	ML
1	250	2020	670	840	70	120	1530	2065	765	960
2	215	650	665	720	75	70	1495	1610	770	770
3	145	740	665	680			1465	1490	770	770
Calculated	1230		815		80		1405		965	

	3α,20β-dihydroxy-5β-pregnane		3β,20β-dihydroxy-5α-pregnane		Δ^4-androstenedione		20α-dihydro-progesterone Acetate*		6β-hydroxy-progesterone Acetate*	
	XL	ML	XL	ML	XL	ML	XL	ML	XL	ML
1	-	-	5490	5905	960	950	2390	2395	2445	2330
2	-	-	5855	5845	960	945	2395	2400	2480	2450
3	-	-								
Calculated	-		6010		960		2520		2370	

*In each of these cases crystal III was mixed with identical crystallized compound isolated from other tissues prior to derivative formation and recrystallization. This accounts for the differences in specific activities between the original compound and its derivative.

elution in which increasing concentrations of ethanol in a changing ratio of methylene chloride in ligroin B was the developing solvent mixture. The elution of radioactivity from the column is shown in Figure 6. The discrete radioactive peaks were pooled and the total cpm in each peak obtained. These data along with the metabolites isolated from the various peaks are given in Table XXIV. More precise identification of the radioactive metabolites was achieved by paper chromatography using the systems shown in Table XXV. The radioactivity eluted from the final paper was mixed with authentic carrier prior to crystallization. Crystallization data for the four metabolites isolated from the kidney extract are shown in Table XXVI.

INTESTINE

The same silica gel column previously described was employed in the isolation of the radioactive metabolites of progesterone from the pooled extracts of the intestines from the perfused previable human fetuses. Radioactivity eluted from the column, determined by counting aliquots from each fraction, is shown in Figure 7.

The fractions under each peak were pooled and counted and the cpm as well as the metabolites isolated are shown in Table XXVII. Further purification of the metabolites was achieved by paper chromatography and the systems used and the radioactivity recovered from the final paper system are shown in Table XXVIII. The amount of authentic carrier added to the labelled metabolite for recrystallization is also shown.

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE KIDNEY FROM THE INTACT FETUS

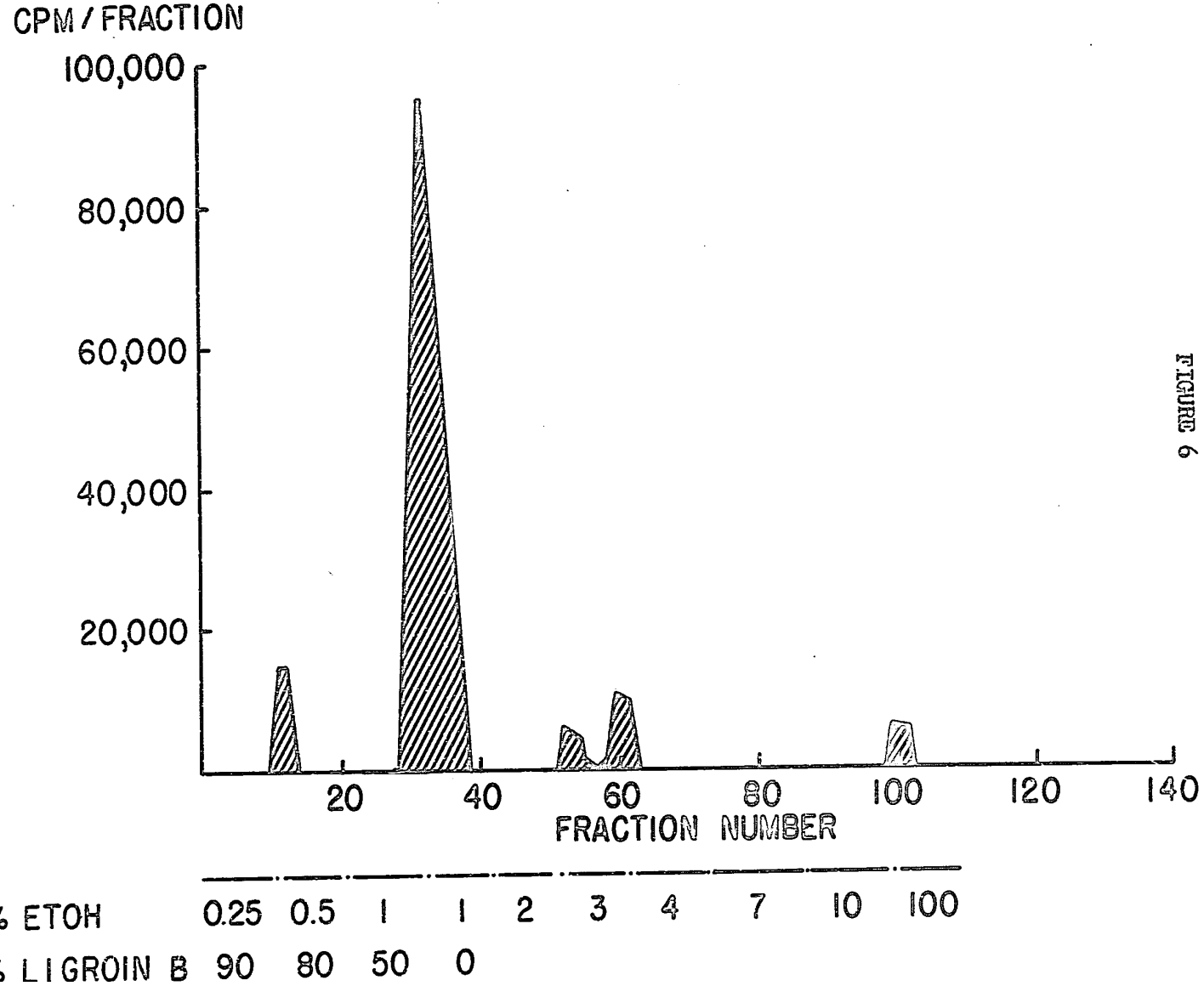

FIGURE 6

TABLE XXIV

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE KIDNEY

Fractions	cpm	Metabolite Isolated
3- 6	22,000	Unknown
31-38	208,000	Progesterone
54-59	15,000	Pregnanolone
60-64	14,000	20α-dihydroprogesterone
70-82	20,000	6β-hydroxyprogesterone 16α-hydroxyprogesterone
90-109	292,000	Three unknowns

TABLE XXV

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE KIDNEY

Metabolite	3β-hydroxy-5α-pregnan-20-one	20α-dihydro-progesterone	6β-hydroxy-progesterone	16α-hydroxy-progesterone
Cpm eluted from silica gel column	15,000	14,000	20,000	20,000
Paper chromatography systems used to purify metabolites	A	C	B ↓ C	B ↓ C
Cpm eluted from final paper	6,750	10,100	6,700	2,500
Carrier steroid (mg) added to eluate	15.25	13.15	11.70	14.00

TABLE XXVI

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT FROM THE KIDNEYS OF THE PERFUSED INTACT FETUS

(specific activities - cpm/mg)

	3β-hydroxy-5α-pregnan-20-one		20α-dihydro-progesterone		6β-hydroxy-progesterone		16α-hydroxy-progesterone	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML
1	250	230	680	800	350	1830	110	350
2	270	280	620	1060	280	700	100	250
3	300	260	630	630	240	720	100	120
4					250	250	100	110
Calculated	300		770		570		180	

	3β,20β-dihydroxy-5α-pregnane		20α-dihydro-progesterone Acetate*		6β-hydroxy-progesterone Acetate*		16α-hydroxy-progesterone Acetate*	
	XL	ML	XL	ML	XL	ML	XL	ML
1	270	240	2390	2400	590	570	435	420
2	270	250	2390	2400	600	600	435	440
Calculated	295		2520		575		440	

*Following crystallization of these metabolites in their free form, the third crystal was mixed with pure crystals obtained with the same metabolite from other tissues prior to derivative formation. This accounts for the discrepancy in specific activity between the free compound and its acetate.

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE INTESTINE FROM THE INTACT FETUS

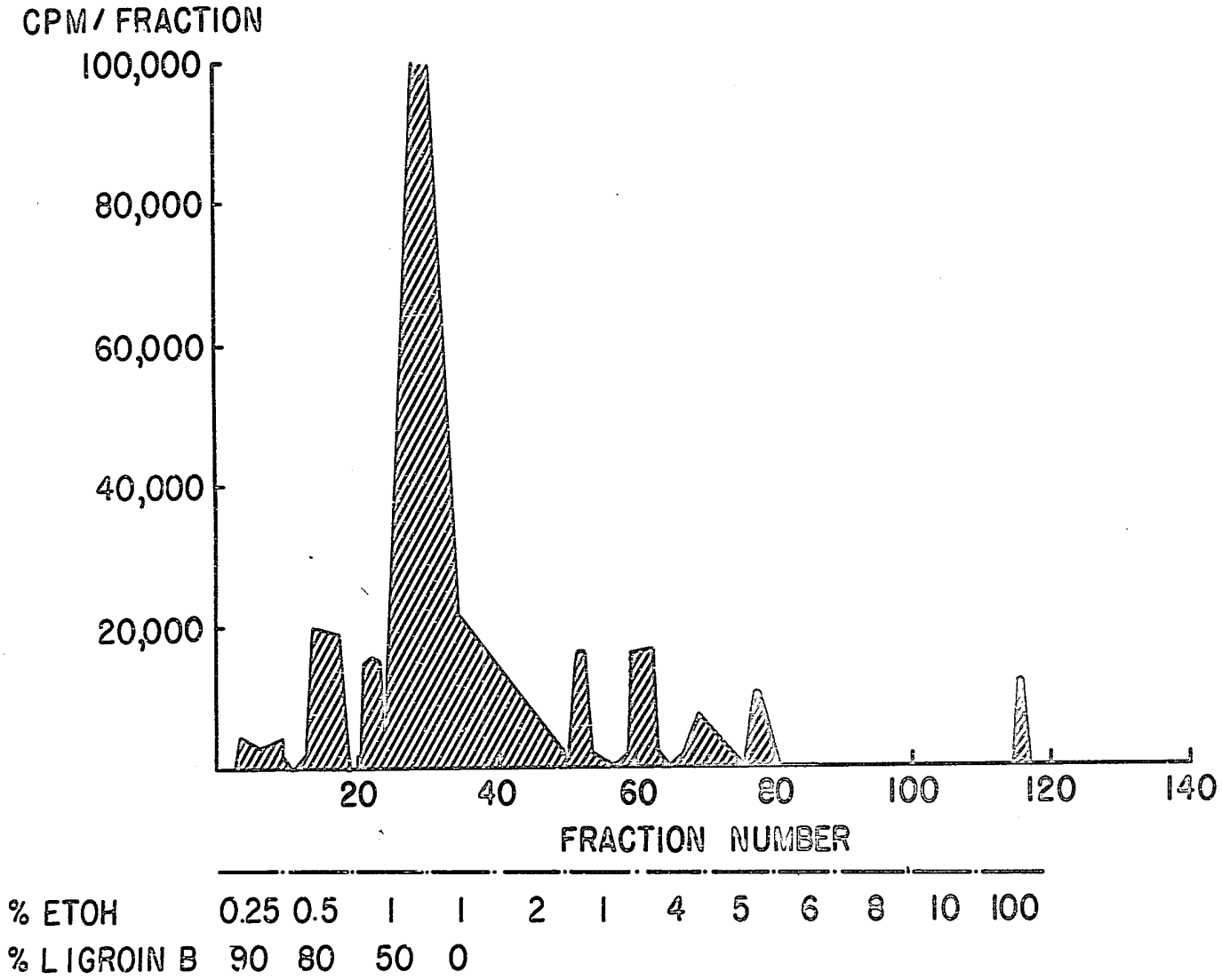

FIGURE 7

TABLE XXVII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE INTESTINE

Fractions	cpm	Metabolite Isolated
5-12	3,000	Unknown
15-22	56,000	Unknown
23-27	45,000	Unknown
28-34	528,000	Progesterone
35-50	153,000	Pregnanolone
51-59	32,000	Pregnanolone
60-67	33,000	20α-dihydroprogesterone
68-76	25,000	Unknown
77-81	18,000	Unknown
96-110	14,000	Unknown
115-118	4,000	Unknown

TABLE XXVIII

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE INTESTINE

Metabolite	Pregnanolone	20α-dihydroprogesterone
Cpm eluted from silica gel column	32,000	33,000
Paper chromatography systems used to purify metabolite	A ↓ B	C ↓ C
Cpm eluted from final paper	16,600	16,450
Carrier steroid (mg) added to eluate	23.40	28.45

Only two compounds were isolated from the extract of intestines and the crystallization data demonstrating the radiochemical purity of these metabolites are shown in Table XXIX.

RESIDUE

After the specific organs had been dissected free, all the tissues left such as the heart, brain, gonads, bone, muscle, and skin were designated as the residue. This extract, accounting for about 15 per cent of all the radioactivity recovered, was chromatographed on a 25 gm silica gel column as previously described. The radioactivity eluted from the column in shown in Figure 8. The fractions composing each peak of radioactivity were pooled and counted and this data is shown in Table XXX. Further purification of the metabolites was carried out using paper chromatography. The systems used, the cpm eluted from the final paper and the weight of carrier added to the partially identified metabolites prior to crystallization are shown in Table XXXI. The data demonstrating radiochemical homogeneity is shown in Table XXXII.

The metabolic activity of the different tissues under investigation is in part reflected by the amount of progesterone isolated from each tissue and also by the amounts of different metabolites produced in these tissues. Using the data presented in Table VII, the total radioactivity in the ether plus aqueous phases can be calculated. This total was taken to represent the radioactivity in each tissue. The proof of identity of each

TABLE XXIX

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE INTESTINE FROM THE PERFUSED INTACT FETUS

(specific activities - cpm/mg)

	Pregnanolone		20α-dihydroprogesterone	
Crystallization	XL	ML	XL	ML
1	305	1925	670	1695
2	310	315	690	690
3	300	300	660	680
Calculated	710		580	
	3α,20β-dihydroxy-5β-pregnane		20α-dihydroprogesterone Acetate	
	XL	ML	XL	ML
1	320	315	580	610
2	322	315	580	570
Calculated	305		585	

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE RESIDUE FROM THE INTACT FETUS

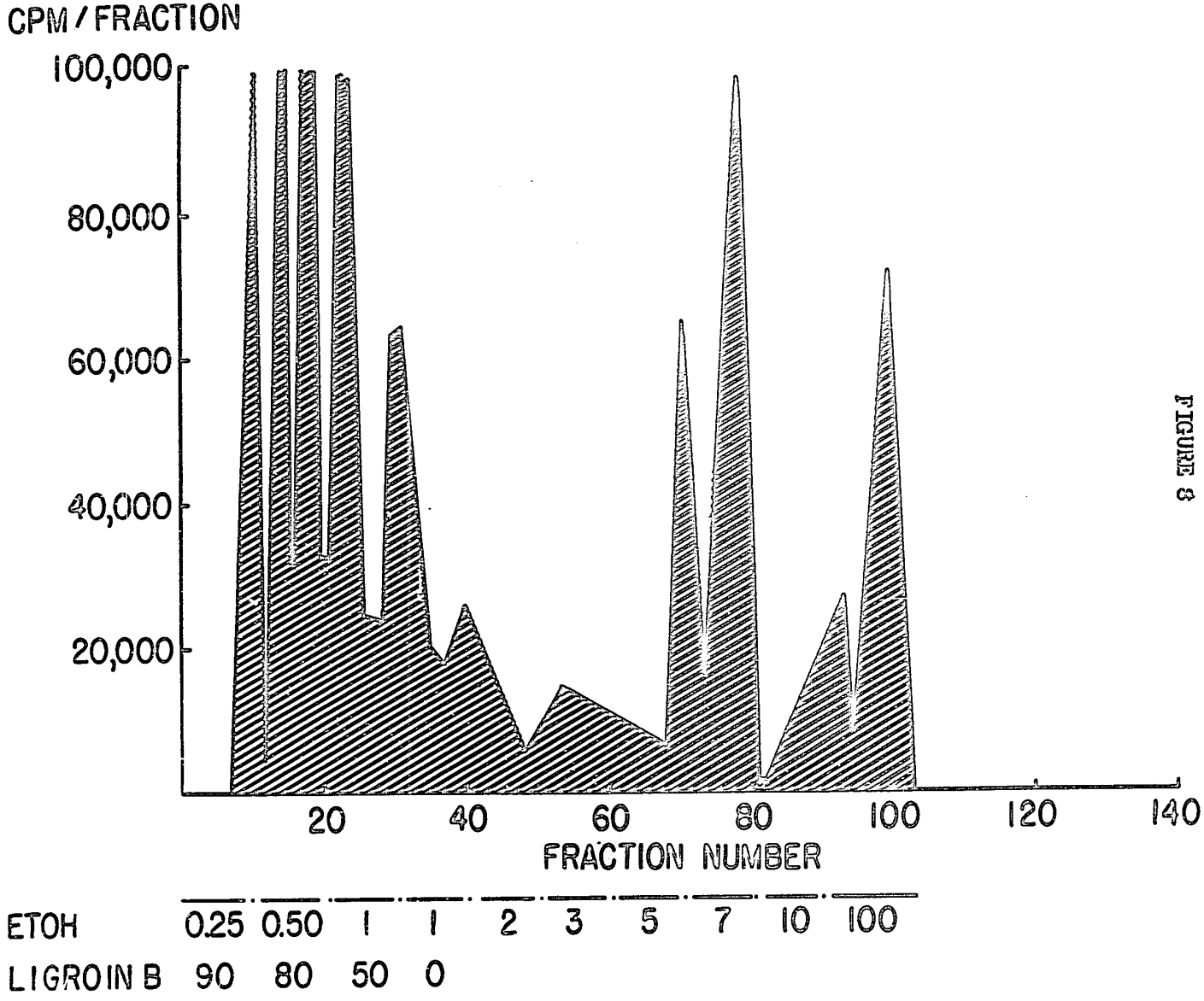

FIGURE 8

TABLE XXX

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE RESIDUE

Fractions	cpm	Metabolite Isolated
2-8	763,000	Unknown
9-13	425,000	Unknown
14-21	4,461,000	Progesterone
22-32	656,000	Pregnanolone
33-38	200,000	20α-dihydroprogesterone plus unknown
39-53	239,000	17α-hydroxyprogesterone 20α-dihydroprogesterone
54-68	134,000	Unknown
69-73	144,000	Three unknowns
76-81	175,000	16α-hydroxyprogesterone plus unknown
82-95	173,000	Two unknowns
96-102	260,000	Unknown

TABLE XXXI

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE RESIDUE

Metabolite	3β-hydroxy-5α-pregnan-20-one	20α-dihydro-progesterone	17α-hydroxy-progesterone	16α-hydroxy-progesterone
Cpm eluted from silica gel column	656,000	199,800	239,000	175,000
Paper chromatography systems used to purify metabolites	A	C	C ↓ A	E ↓ C
Cpm eluted from final paper	454,500	34,800	47,900	25,600
Carrier steroid (mg) added to eluate	32.60	18.85	43.05	36.35

TABLE XXXII

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT FROM THE RESIDUE OF THE PERFUSED INTACT FETUS

(specific activities - cpm/mg)

	3β-hydroxy-5α-pregnan-20-one		20α-dihydro-progesterone		17α-hydroxy-progesterone		16α-hydroxy-progesterone	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML
1	10540	24950	1330	2350	1080	1190	560	820
2	10870	11520	1320	1660	1050	1070	590	660
3	10430	10990	1320	1320			600	590
Calculated	13940		1850		1110		690	
	3β,20β-dihydroxy-5α-pregnane*		20α-dihydro-progesterone Acetate*		Δ^4-andro-stenedione		16α-hydroxy-progesterone Acetate*	
	XL	ML	XL	ML	XL	ML	XL	ML
1	5890	5900	2390	2400	960	950	430	420
2	5860	5850	2390	2400	960	950	430	440
Calculated	6010		2520		960		440	

*Following successful crystallization of the metabolite, crystal III was mixed with the crystal III of the same compound in other tissues. The derivative was then formed and recrystallized. This accounts for the difference in specific activities between the derivative and its parent compound.

metabolite was established by recrystallization to constant specific activity expressed as cpm per mg. If the specific activity (cpm/mg) of the final crystal, prior to derivative formation, is multiplied by the weight of carrier (mg) added, then the amount of radioactivity (cpm) in the specific metabolite can be calculated. The percentage of the total radioactivity in a tissue represented by this purified metabolite is then calculated. In Table XXXIII the percentage of unchanged progesterone and of the radioactive metabolites found in each tissue are shown. This is a minimal figure as there is no correction for losses incurred during the procedures.

TABLE XXXIII

UNCONJUGATED METABOLITES ISOLATED FROM THE TISSUES OF THE INTACT MIDTERM HUMAN FETUS PERFUSED WITH PROGESTERONE (EXPRESSED AS PERCENTAGE OF TOTAL ETHER AND WATER SOLUBLE RADIOACTIVITY)

	ADRENAL	PERFUSATE	LIVER	LUNG	KIDNEY	INTESTINE	RESIDUE
Progesterone	4.2	9.5	2.9	35.2	43.3	28.3	26.6
Pregnanolone	-	< 0.5	4.0	-	-	0.6	-
3β-hydroxy-5α-pregnan-20-one	-	< 0.5	-	1.0	1.2	-	3.8
20α-dihydroprogesterone	2.0	1.1	9.0	1.3	2.1	1.5	< 0.5
Pregnanediol	-	-	20.7	-	-	-	-
6β-hydroxyprogesterone	-	< 0.5	-	0.8	0.8	-	-
16α-hydroxyprogesterone	2.8	1.7	-	-	0.5	-	< 0.5
17α-hydroxyprogesterone	0.6	-	-	< 0.5	-	-	0.5
Corticosterone	-	< 0.5	-	-	-	-	-
Cortisol	0.6	-	-	-	-	-	-

(- not isolated)

ETHER SOLUBLE METABOLITES ISOLATED FROM THE ADRENALECTOMIZED FETUSES

GENERAL

Following the partitioning of the tissue extract between ether and water, the aqueous phase was evaporated to dryness. The residue was dissolved in methanol and stored in the refrigerator until processed. The ether fractions were taken to dryness and transferred to weighed tubes in preparation for purification and isolation of metabolites.

The initial step in the separation of the metabolites of the ether extracts was by silica gel column chromatography as described in the previous section. Fractions under discrete peaks of radioactivity were pooled and further purified by paper chromatography. When a tentative identification of a metabolite was made by comparing its mobility in different paper chromatographic systems with known standards, proof of its radiochemical homogeneity was provided by crystallization to constant specific activity as previously described.

This procedure of purification, isolation, and proof of radiochemical purity was carried out for each metabolite of 4-^{14}C-progesterone isolated from the tissues to be described below.

PERFUSATE

The perfused blood was collected from the umbilical vein catheter(s) during four fifteen minute intervals which are referred to as perfusates I to IV. Each perfusate was processed separately.

Initial silica gel chromatography was carried out using several solvent changes at times indicated by the radioactive material detectable in the fractions. An increasing concentration of ethanol in an increasing ratio of methylene chloride to ligroin B served to obtain some separation and purification of the metabolites. Figure 9 shows the pattern of elution of radioactivity when the perfusate collected from 45 to 60 min. was chromatographed on silica gel. This pattern is typical of that for all four perfusates.

The selection of the column fractions to be pooled together was controlled by the elution pattern of the radioactive material. The fractions chosen, the cpm in each pool, and the compound(s), if any, isolated from the pool(s) are shown in Tables XXXIV, XXXV, XXXVI, and XXXVII.

A total of four metabolites in addition to progesterone were found in the extracts of the four perfusate samples collected from the adrenalectomized fetuses. The sequential paper chromatographic systems and the cpm eluted from the final paper chromatogram are shown in Table XXXVIII. The weight of authentic non-radioactive

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE PERFUSATE FROM THE ADRENALECTOMIZED FETUS

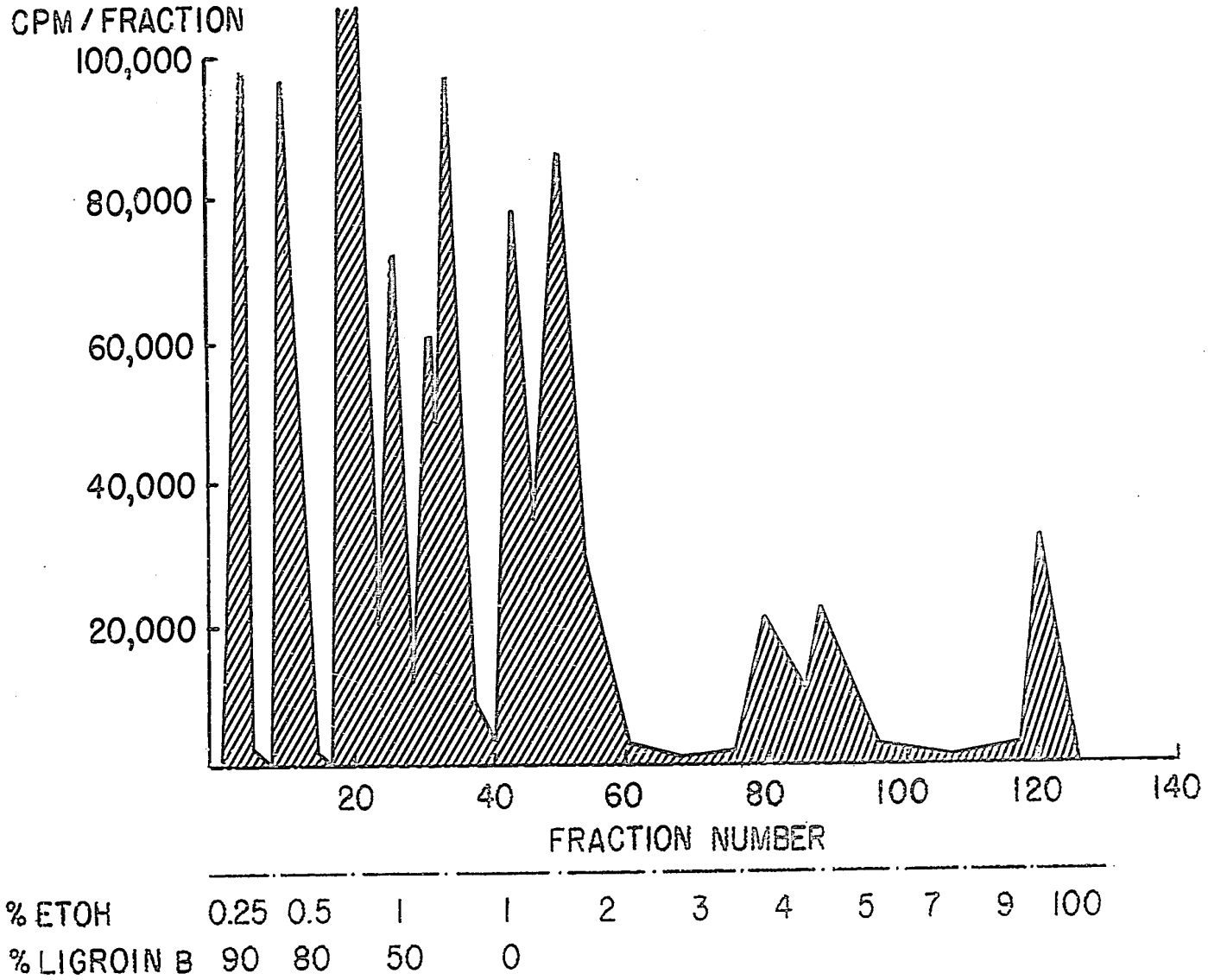

FIGURE 9

TABLE XXXIV

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE I

Fractions	cpm	Metabolite Isolated
3-12	40,000	Unknown
23-32	5,120,000	Progesterone
33-36	66,000	Progesterone
37-57	91,000	Progesterone
58-62	23,000	Unknown
63-72	90,000	20α-dihydroprogesterone
73-86	334,000	Two unknown
87-120	34,000	Three unknown
121-126	9,000	One unknown

TABLE XXXV

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE II

Fractions	cpm	Metabolite Isolated
4-15	328,000	Unknown
16-25	6,230,000	Progesterone
26-36	320,000	Progesterone, Pregnanolone
37-48	165,000	Progesterone, Pregnanolone
49-57	10,000	Progesterone
58-70	277,000	Pregnanolone, 20α-dihydroprogesterone
71-79	421,000	6β-hydroxyprogesterone plus unknown
80-92	81,000	Two unknown
93-101	38,000	Two unknown
102-113	35,000	Two unknown
114-119	42,000	Unknown

TABLE XXXVI

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE III

Fractions	cpm	Metabolite Isolated
3-11	267,000	Unknown
12-21	5,263,000	Progesterone
22-25	58,000	Progesterone
26-36	124,000	Pregnanolone, Progesterone
37-40	30,000	Pregnanolone
41-64	332,000	20α-dihydroprogesterone
65-76	372,000	Pregnanediol, plus unknown
77-91	100,000	Two unknown
92-100	36,000	Two unknown
101-112	54,000	Two unknown
113-119	44,000	One unknown

TABLE XXXVII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM PERFUSATE IV

Fractions	cpm	Metabolite Isolated
4- 9	119,000	Unknown
10-15	220,000	Unknown
16-22	4,325,000	Progesterone
23-27	90,000	Progesterone, Pregnanolone
28-31	180,000	Progesterone, Pregnanolone
32-38	245,000	Progesterone, Pregnanolone
39-43	171,000	20α-dihydroprogesterone
44-53	267,000	20α-dihydroprogesterone
74-83	185,000	Pregnanediol, plus unknown
84-90	124,000	One unknown
121-126	96,000	Three unknown

TABLE XXXVIII

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE PERFUSATE

	Progesterone	Pregnanolone	20α-dihydro-progesterone	Pregnanediol	6β-hydroxy-progesterone
Cpm eluted from silica gel column	4,325,000	180,000	267,000	124,000	184,000
Paper chromatography systems used to purify metabolites	A ↓ B	B ↓ A	A ↓ C	E ↓ B ↓ A	B ↓ C
Cpm eluted from final paper	3,189,000	52,500	215,850	58,100	72,215
Carrier steroid (mg) added to eluate	196.12	52.60	21.10	38.40	33.55

carrier steroid added to the radioactive material for subsequent crystallization is also shown in this table.

The data showing that radiochemical homogeneity was achieved for each compound is shown in Table XXXIX. Unfortunately the occurrence of the pregnanolone epimer was not recognized at the time these results were obtained. The excess counts in the first mother liquor resulting from the crystallization of the radioactive material with pregnanolone suggests that some of the excess counts may represent the epimer, 3β-hydroxy-5α-pregnan-20-one.

LIVER EXTRACT

The procedure for the isolation of progesterone and its ether soluble metabolites from the liver extract of the perfused adrenalectomized fetuses involved the use of silica gel absorption column chromatography as described earlier. The graphic illustration of the radioactive material eluted from the column was obtained by plotting the cpm in each fraction against the individual fractions, as shown in figure 10.

The discrete peaks of radioactive material were pooled as shown in Table XL and the total cpm and compound(s) isolated from the various peaks are also shown in this table.

Further purification and tentative identification of the metabolites was gained by the use of sequential paper chromatography using the systems shown in Table XLI. The cpm eluted from the final paper and the weight of non-radioactive carrier steroid mixed with

TABLE XXXIX

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT FROM THE FOUR PERFUSATES OF THE PERFUSED ADRENALECTOMIZED HUMAN FETUS

(specific activities - cpm/mg)

	Progesterone		20α-dihydro-progesterone		Pregnanediol		Pregnanolone		6β-hydroxy-progesterone	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML	XL	ML
1	15700	16100	10100	9800	1390	1410	330	6400	1745	3065
2	15600	15200	10500	10200	1400	1400	310	1000	2060	3535
3	15900	16000	10500	10500			285	405	2050	2370
4							290	285	2020	2180
5							290	285		
Calculated	16300		10200		1500		1000		2245	

	Δ^4-pregnen-3β,20β-diol		20α-dihydro-progesterone Acetate		Pregnanediol Diacetate		3α,20β-dihydroxy-5β-pregnane		6β-hydroxy-progesterone Acetate	
	XL	ML	XL	ML	XL	ML	XL	ML	XL	ML
1	15400	15100	9500	7900	1110	1010	280	275	1715	1790
2	14900	15100	9400	9000	1110	1100	280	295	1750	1795
Calculated	15800		9300		1100		290		1790	

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE LIVER FROM THE ADRENALECTOMIZED FETUS

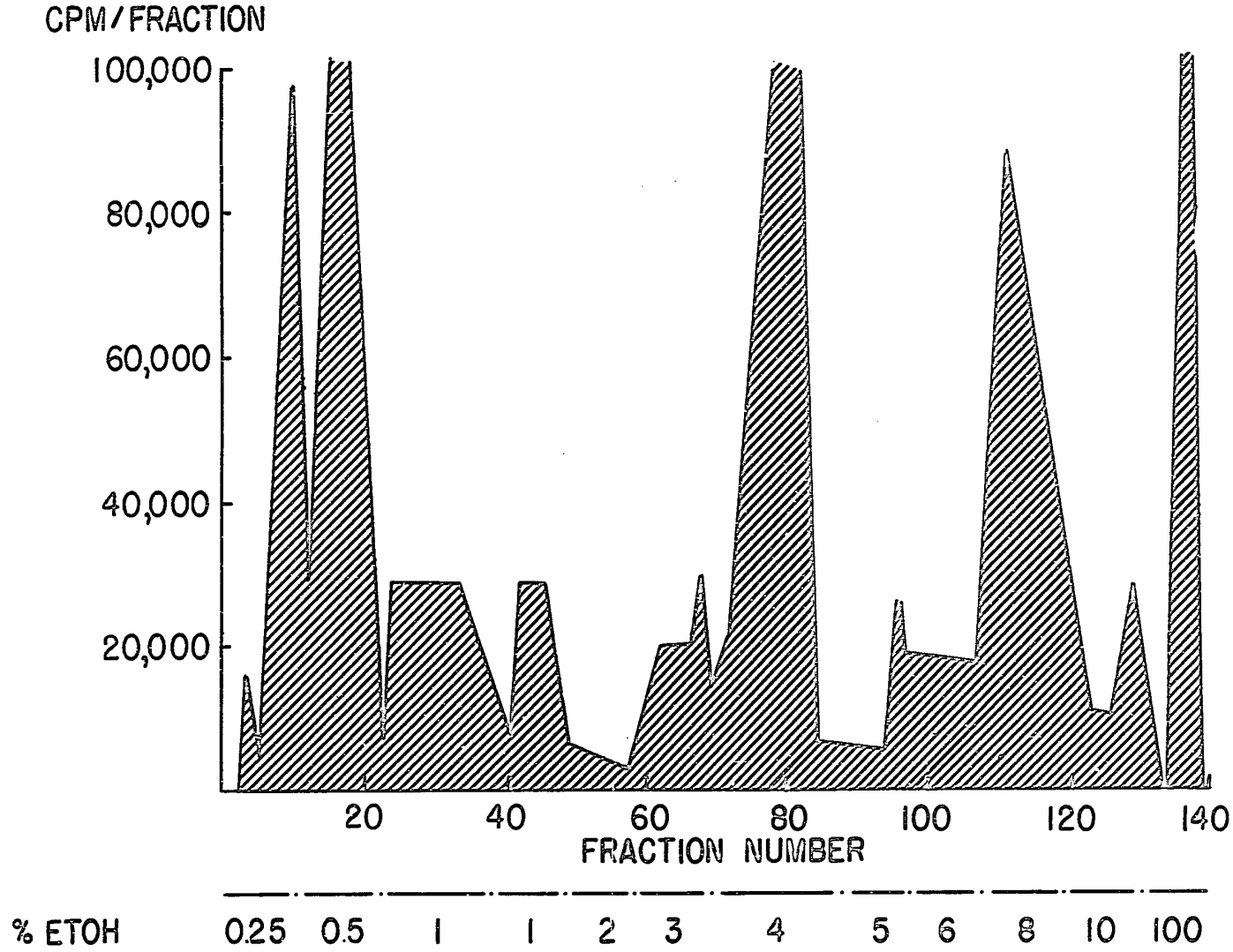

FIGURE 10

TABLE XL

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE LIVER

Fractions	cpm	Metabolite Isolated
5- 9	54,000	Unknown
10-13	285,000	Progesterone
14-24	654,000	Pregnanolone, Progesterone
25-41	333,000	20α-dihydroprogesterone
42-62	181,000	20α-dihydroprogesterone plus one unknown
63-74	226,000	Three unknowns
75-95	2,418,000	Pregnanediol plus unknown
96-109	161,000	Unknown
110-122	431,000	Unknown
123-135	88,000	Unknown
136-140	283,000	Unknown

TABLE XLI

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE LIVER

Metabolite	Pregnanolone	20α-dihydroprogesterone	Pregnanediol
Cpm eluted from silica gel column	654,000	333,000	2,418,000
Paper chromatography systems used to purify metabolites	B → A	B → A → B	A → B
Cpm eluted from final paper	304,450	174,600	1,624,700
Carrier steroid (mg) added to eluate	89.75	53.85	72.50

the radioactivity is shown in this table.

The crystallization results which established the identity and degree of purity of these radioactive metabolites are shown in Table XLII.

LUNG EXTRACT

The ether soluble portion of the extract from the lungs of the adrenalectomized perfused fetuses was chromatographed on a 25 gram silica gel column in the manner previously described. In figure 11 the plot of the cpm per fraction against the fraction number is shown. The solvent changes are also shown.

The discrete peaks of radioactive material were pooled and counted as shown in Table XLIII. Compounds that were completely identified from the various peaks are also shown.

Tentative identification was obtained by descending paper chromatography using appropriate systems as shown in Table XLIV. For recrystallization the eluted radioactive material was mixed with authentic carrier steroid as shown in this table.

The crystallization data for the three compounds are shown in Table XLV.

TABLE XLII

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE LIVER FROM PERFUSED ADRENALECTOMIZED FETUSES

(specific activities - cpm/mg)

	Pregnanolone		20α-dihydroprogesterone		Pregnanediol	
Crystallization	XL	ML	XL	ML	XL	ML
1	2110	4640	2990	3730	22400	26400
2	2060	2460	3070	3010	22900	22100
3	2060	2350	3030	3040	22600	22400
4	2090	2090				
Calculated	3390		3230		22400	
	3α,20β-dihydroxy-5β-pregnane		20α-dihydroprogesterone Acetate		Pregnanediol Diacetate	
	XL	ML	XL	ML	XL	ML
1	1980	1980	2470	2470	18400	20200
2	1970	1970	2470	2460	18400	18600
Calculated	2080		2670		17900	

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE LUNG FROM THE ADRENALECTOMIZED FETUS

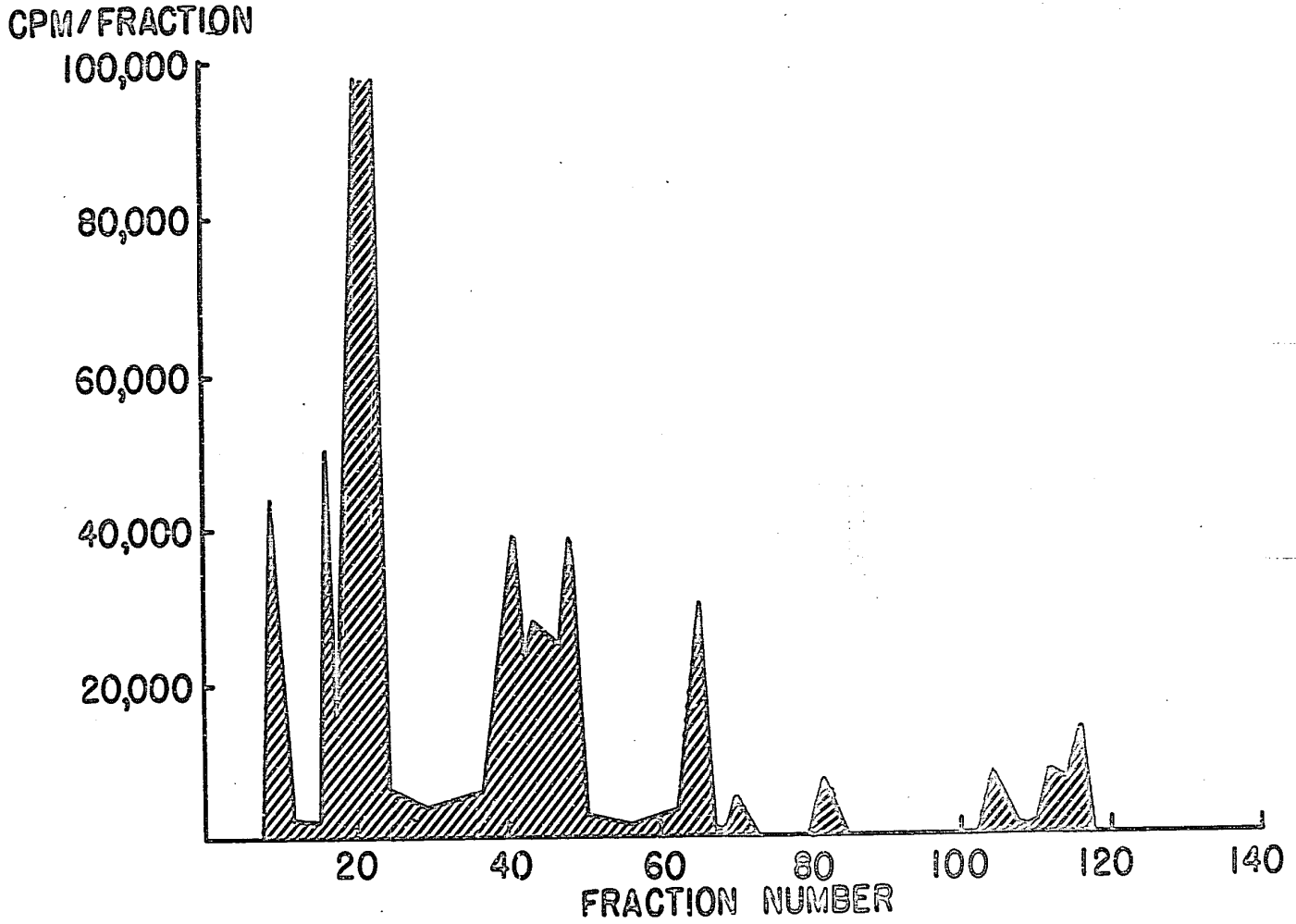

FIGURE 11

TABLE XLIII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE LUNG

Fractions	cpm	Metabolite Isolated
3- 9	68,000	Unknown
10-18	128,000	Unknown
19-25	913,000	Progesterone
26-35	46,000	Progesterone, Pregnanolone
36-42	76,000	Pregnanolone
43-54	105,000	Pregnanolone
55-69	56,000	20α-dihydroprogesterone
70-76	16,000	6β-hydroxyprogesterone plus unknown
77-88	22,000	Unknown
89-112	38,000	Unknown
113-119	16,000	Unknown

TABLE XLIV

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE LUNG

Metabolite	3β-hydroxy-5α-pregnan-20-one	20α-dihydro-progesterone	6β-hydroxy-progesterone
Cpm eluted from silica gel column	76,000	56,000	16,000
Paper chromatography systems used to purify metabolites	A	C	C
Cpm eluted from final paper	49,700	39,800	7,100
Carrier steroid (mg) added to eluate	39.40	19.95	9.20

TABLE XLV

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE LUNG FROM THE PERFUSED ADRENALECTOMIZED FETUSES

(specific activities - cpm/mg)

	3β-hydroxy-5α-pregnan-20-one		20α-dihydroprogesterone		6β-hydroxyprogesterone	
Crystallization	XL	ML	XL	ML	XL	ML
1	1100	1730	1760	2760	545	2370
2	1130	1120	1760	1830	555	540
3	1100	1100	1790	1760	535	575
Calculated	1260		1990		770	
	3β,20β-dihydroxy-5α-pregnane*		20α-dihydroprogesterone Acetate*		6β-hydroxyprogesterone Acetate*	
	XL	ML	XL	ML	XL	ML
1	760	780	3170	3200	2440	2230
2	770	780	3150	3190	2480	2450
Calculated	800		3420		2370	

*Following successful crystallization of the metabolites, crystal III was mixed with the same metabolite crystallized from other tissues. This mixture was then subjected to derivative formation and this accounts for the discrepancies in specific activities between the free form and its derivative.

KIDNEY EXTRACT

A silica gel absorption column was employed for the first step in purifying the ether soluble kidney extract using the system described previously. In figure 12, the radioactivity eluted has been plotted against the fraction number.

The individual peaks of radioactive material, found by counting aliquots of the fractions, were pooled together and recounted. These data are shown in Table XLVI. Only two metabolites were isolated from this extract. The further purification by paper chromatography is indicated in Table XLVII. The cpm eluted from the paper and the weight of carrier steroid mixed with it for crystallization are also shown in Table XLVII. The data demonstrating the radiochemical purity are given in Table XLVIII.

INTESTINAL EXTRACT

The relatively simple chromatogram obtained when the ether soluble extract from the adrenalectomized fetuses was chromatographed is shown in figure 13. This was absorption column chromatography on a 25 gram silica gel column eluted with increasing concentrations of ethanol in methylene chloride and ligroin B.

Pooling of the fractions was carried out as shown in Table XLIX and the total counts in the peak along with any compound isolated from that peak are shown in the same table. The radioactive pools were studied further on suitable paper chromatographic

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE KIDNEY FROM THE ADRENALECTOMIZED FETUS

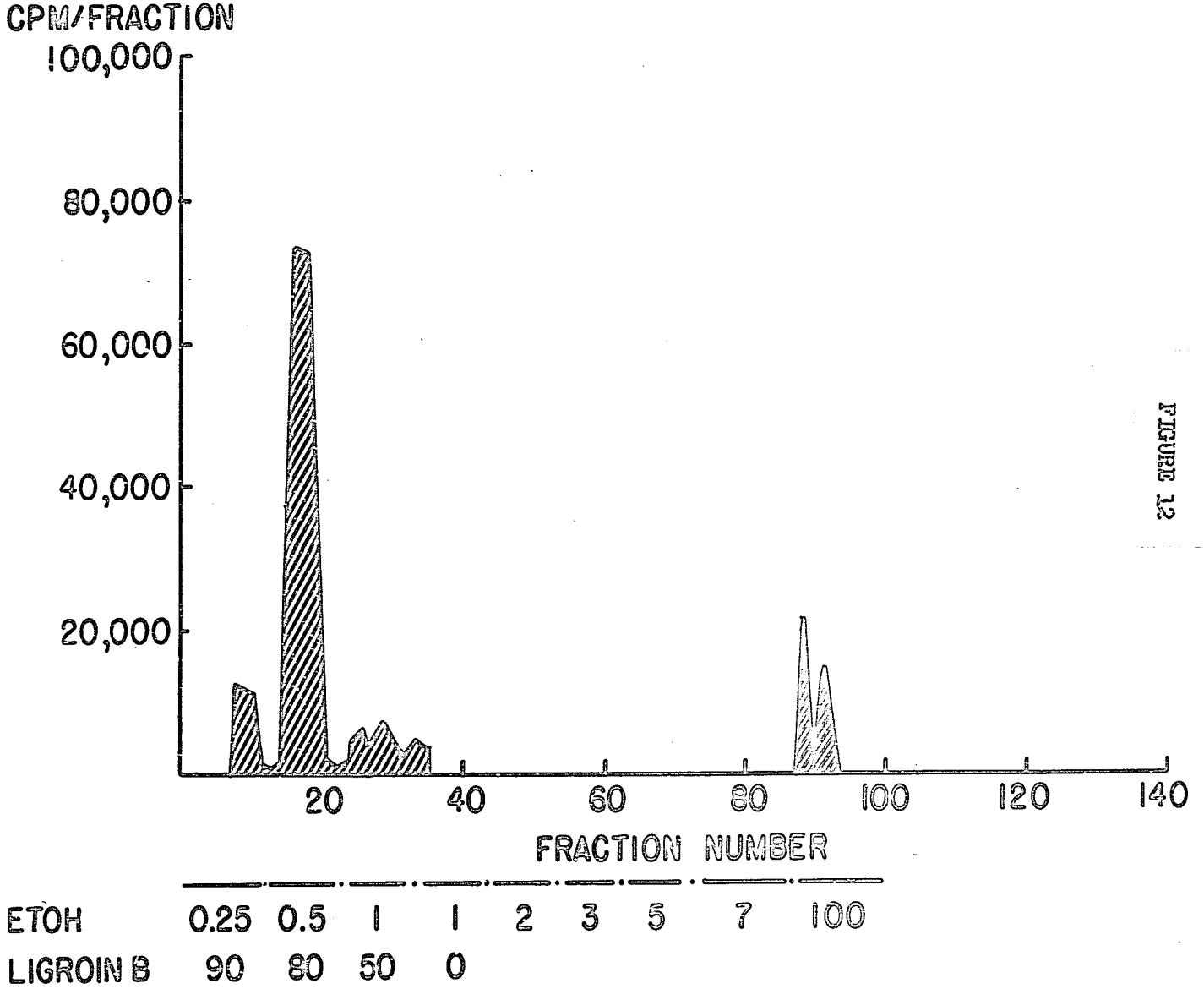

FIGURE 12

TABLE XLVI

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE KIDNEY

Fractions	cpm	Metabolite Isolated
4- 6	11,000	Unknown
7-11	18,000	Unknown
17-22	152,000	Progesterone
23-25	4,000	Progesterone, Pregnanolone
26-31	13,000	Pregnanolone
32-48	30,000	20α-dihydroprogesterone pregnanolone
70-73	10,000	Two unknown
90	1,000	Unknown
92-96	10,000	Unknown

TABLE XLVII

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE KIDNEY

Metabolite	3β-hydroxy-5α-pregnan-20-one		20α-dihydroprogesterone
Cpm eluted from silica gel column	13,000	30,000	30,000
Paper chromatography systems used to purify metabolites	A	C	C
Cpm eluted from final paper	6,000 → 25,000 ← 19,000		4,600
Carrier steroid (mg) added to eluate	31.30		11.0

TABLE XLVIII

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT FROM THE KIDNEY OF THE PERFUSED ADRENALECTOMIZED FETUS

(specific activities - cpm/mg)

	3β-hydroxy-5α-pregnan-20-one		3β,20β-dihydroxy-5α-pregnane*		20α-dihydro-progesterone		20α-dihydro-progesterone Acetate*	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML
1	530	820	760	780	380	540	3170	3200
2	500	570	770	780	390	400	3150	3180
3	510	510			380	370		
Calculated	800		800		420		3420	

*The crystalline material obtained in the third crystallization was mixed with similar material isolated from other tissues and then a suitable derivative was formed and crystallized. This step accounts for the difference beyond that expected in the specific activities of the original compound and its derivatives.

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE INTESTINE FROM THE ADRENALECTOMIZED FETUS

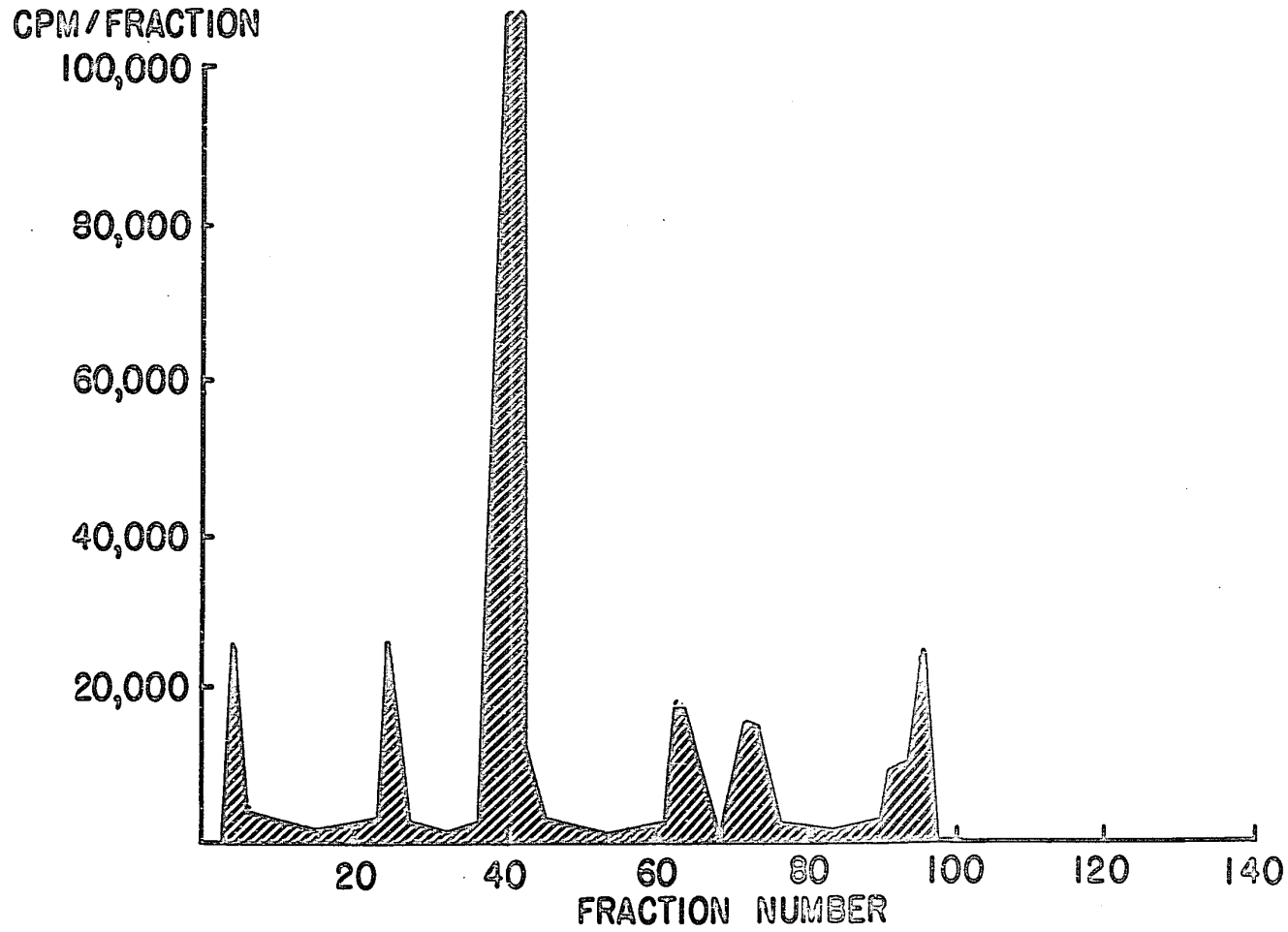

FIGURE 13

TABLE XLIX

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE INTESTINE

Fractions	cpm	Metabolite Isolated
3- 9	48,000	Unknown
25-32	61,000	Progesterone
38-46	645,000	Progesterone
47-61	60,000	Pregnanolone
62-69	48,000	Pregnanolone
71-76	44,000	20α-dihydroprogesterone
88-96	66,000	Two unknown

systems. The amount of radioactivity recovered from the final paper and the weight of non-radioactive steroid mixed with it for crystallization is shown in Table L.

That radiochemical homogeneity was achieved by crystallization of the two compounds is shown in Table LI.

EXTRACT OF RESIDUE

The residue of the fetus was considered to include all tissues remaining after careful dissection and removal of the individual organs being studied separately. Included in the residue would be the brain, heart, gonads, muscle, bone and skin. The adrenals were removed prior to any perfusion of labelled 4-^{14}C-progesterone. Following the ether:aqueous partition, the ether soluble material was chromatographed on a 25 gram silica gel column. The column was developed using a discontinuous gradient with an increasing concentration of absolute ethanol in an increasing ratio of methylene chloride to ligroin B. Aliquots were taken from each 5 ml fraction and the radioactivity in each aliquot measured. The cpm per fraction were plotted against the fraction number as shown in figure 14.

The peaks of radioactivity were pooled and total cpm obtained as in Table LII. Further purification and initial identification of the radioactive metabolites were carried out using paper chromatography as outlined in Table LIII. The cpm eluted from the final paper are also shown. In the case of pregnanolone only a portion

TABLE L

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE INTESTINE

Metabolite	Pregnanolone	20α-dihydroprogesterone
Cpm eluted from silica gel column	60,000	44,000
Paper chromatography systems used to purify metabolites	A ↓ B	C ↓ B
Cpm eluted from final paper	36,700	23,500
Carrier steroid (mg) added to eluate	28.60	28.40

TABLE LI

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE INTESTINE OF THE PERFUSED ADRENALECTOMIZED FETUS

(specific activities - cpm/mg)

	Pregnanolone		3α,20β-dihydroxy-5β-pregnane		20α-dihydro-progesterone		20α-dihydro-progesterone Acetate	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML
1	570	6360	550	550	770	870	600	670
2	550	1240	560	550	780	790	660	670
3	550	550			740	740		
Calculated	1270		545		830		660	

SILICA GEL COLUMN CHROMATOGRAPHY OF THE ETHER EXTRACT OF THE RESIDUE FROM THE ADRENALECTOMIZED FETUS

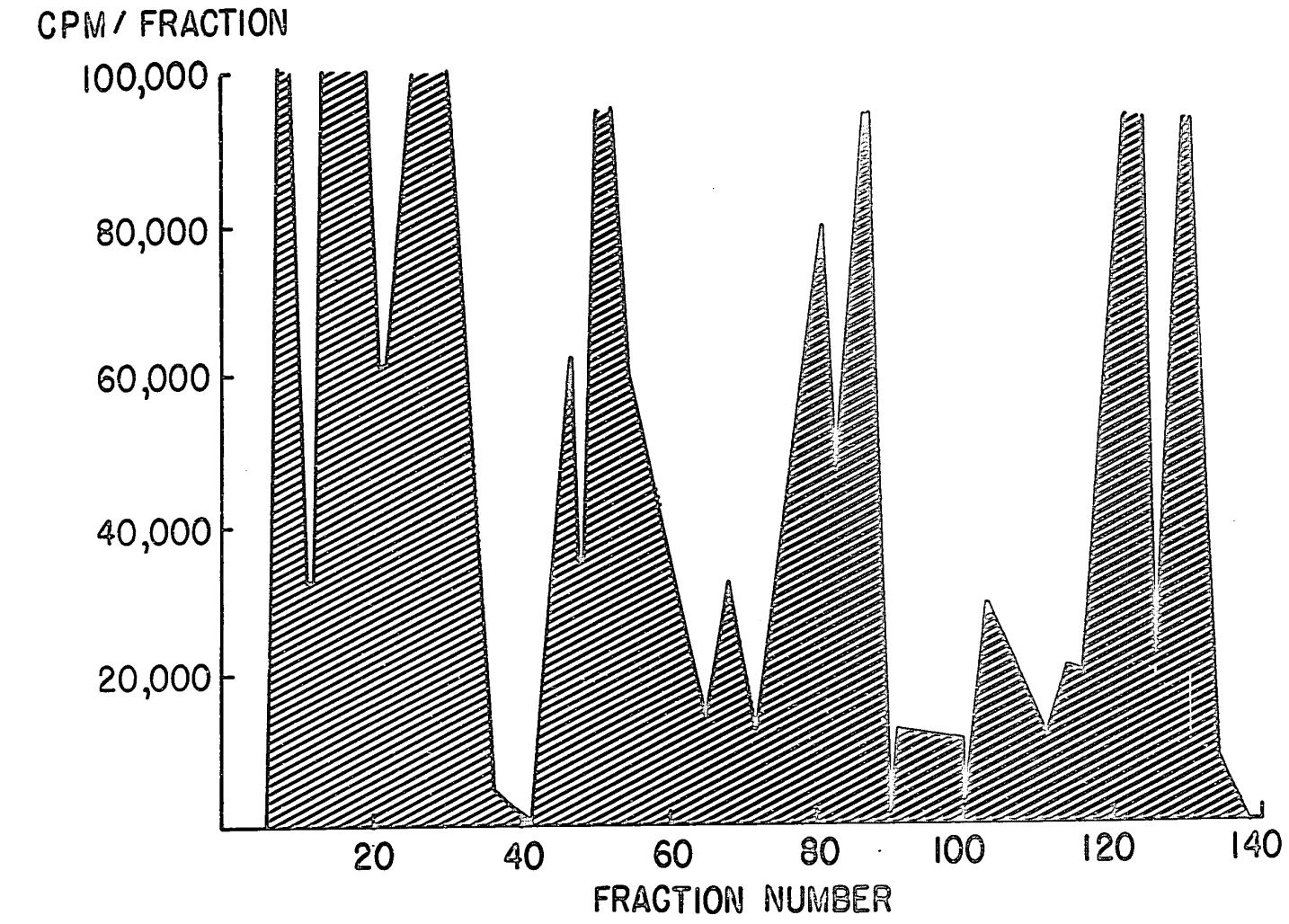

FIGURE 14

TABLE LII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE ETHER FRACTION FROM THE RESIDUE

Fractions	cpm	Metabolite Isolated
3- 5	1,932,000	Unknown
6-10	1,344,000	Unknown
11-23	7,433,000	Progesterone
24-38	2,173,000	Progesterone, Pregnanolone
43-48	171,000	Progesterone, Pregnanolone plus unknown
49-66	1,074,000	20α-dihydroprogesterone, 17α-hydroxyprogesterone
67-75	151,000	20α-dihydroprogesterone, 17α-hydroxyprogesterone
76-87	470,000	Three unknown
88-95	434,000	Two unknown
96-102	54,000	Three unknown
103-111	133,000	Two unknown
112-117	93,000	Two unknown
118-126	516,000	One unknown
127-137	284,000	One unknown

TABLE LIII

PURIFICATION OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE RESIDUE

Metabolite	3β-hydroxy-5α-pregnan-20-one	20α-dihydro-progesterone	17α-hydroxy-progesterone
Cpm eluted from silica gel column	2,173,000	1,073,000	1,073,000
Paper chromatography systems used to purify metabolites	A	C ↓ A	C ↓ A
Cpm eluted from final paper	1,795,000 (85,000)*	270,000	205,700
Carrier steroid (mg) added to eluate	34.0	61.11	64.20

*Only 85,000 cpm were mixed with the 34.0 mg of carrier steroid for crystallization

(85,000) of the total recovered radioactivity was used to mix with the appropriate weight of nonradioactive carrier steroid as shown. This mixture was then crystallized, subjected to derivative formation and then recrystallized to demonstrate the proof of identity and degree of purity of the isolated metabolite. These data are shown in Table LIV. As with all tissue metabolites, the infra-red spectrum of the product of derivative formation was obtained as verification of its structure.

As an indirect guide to the metabolic activity of the various tissues in the perfused midterm previable human fetus in the absence of both adrenal glands, the per cent yield of progesterone metabolites in the different tissues has been calculated. This is based on the total cpm found in the ether and aqueous phases after partition, using the data from Table VIII. The total cpm thought to represent purified metabolite was obtained by multiplying the specific activity (cpm/mg) of the final crystal by the weight (mg) of the authentic carrier steroid used in the crystallization. The fraction of the total radioactivity in a tissue found as a radioactive metabolite in that tissue has been expressed as a percentage in Table LV.

TABLE LIV

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE ETHER EXTRACT OF THE RESIDUE OF THE ADRENALECTOMIZED FETUS

(specific activities - cpm/mg)

	3β-hydroxy-5α-pregnan-20-one		20α-dihydro-progesterone		17α-hydroxy-progesterone	
Crystallization	XL	ML	XL	ML	XL	ML
1	2380	4150	4520	4860	3420	3490
2	2170	2650	4520	4750	3460	3460
3	2240	2280	4290	4260		
4	2200	2250				
Calculated	2500		4400		3200	

	3β,20β-dihydroxy-5α-pregnane		20α-dihydro-progesterone Acetate		Δ^4-androstenedione	
	XL	ML	XL	ML	XL	ML
1	2130	2220	3850	3710	3540	3530
2	2120	2180	3860	3740	3550	3520
Calculated	2210		3780		3685	

TABLE LV

UNCONJUGATED METABOLITES OF PROGESTERONE ISOLATED FROM THE VARIOUS TISSUES OF THE PERFUSED, MIDTERM PREVIABLE, ADRENALECTOMIZED FETUS (EXPRESSED AS PERCENTAGE OF TOTAL ACTIVITY IN THE TISSUE FOUND AS ONE METABOLITE)

	Perfusate	Liver	Lung	Kidney	Intestine	Residue
Progesterone	34.1	3.2	46	43.7	44.4	29.2
Pregnanolone	< 0.5	2.8	-	-	1.3	-
3β-hydroxy-5α-pregnan-20-one	-	-	2.6	0.5		< 0.5
20α-dihydroprogesterone	2.4	2.4	2.1	1.3	1.7	1.4
Pregnanediol	-	24.0	-	-	-	-
6β-hydroxyprogesterone	0.7	-	< 0.5	-	-	-
17α-hydroxyprogesterone	-	-	-	-	-	1.2

- Not isolated

ISOLATION OF CONJUGATED METABOLITES FROM THE TISSUES OF THE INTACT AND ADRENALECTOMIZED PERFUSED PREVIABLE HUMAN FETUS

The data obtained from the ether-aqueous partition indicated that many of the tissues had very small amounts of radioactive material in the aqueous fraction. The procedure utilized for the separation, isolation and identification of metabolites was more complex than that used with the ether fractions and each step was associated with unavoidable losses. Thus the isolation of metabolites from tissue extracts having low total amounts of radioactivity was precluded and so as a result only selected tissue extracts were processed. In the experiments involving the intact, perfused fetus, the extracts from adrenal, liver, and residue were studied, and in the adrenalectomized fetus only liver and residue.

Initial purification of the metabolites in the aqueous extract was achieved using a Celite partition column in which the stationary phase was ammonium hydroxide:water (25:475) previously equilibrated with the mobile phase which was iso-octane:t-butanol (300:500). The column was "wet-packed" as described in "Methods" and after the mobile phase had percolated through the column for twelve hours, the sample was applied in a slurry of Celite by the same "wet-pack" technique. The column was developed with mobile phase and 10 ml fractions were collected. An aliquot from each fraction was used to determine the total cpm in the fraction. The polarity of the mobile phase was increased after the first forty fractions had been collected by changing the ratio of solvents in the mobile phase to

iso-octane:t-butanol (200:500). The next solvent change was made after 140 to 180 fractions had been collected and the ratio was changed to iso-octane:t-butanol (150:500). The final solvent change prior to stripping the column with absolute ethanol was to iso-octane: t-butanol (100:500) and occurred after about 200 fractions had been collected.

Material eluted as discrete peaks of radioactivity from the column was then subjected to further purification by paper partition chromatography using the solvent systems shown in Table VI. Due to the lack of carrier amounts of conjugates it was frequently impossible to make a tentative identification of the metabolites by comparison of mobilities on paper. In these instances the unknowns were subjected to hydrolysis with β-glucuronidase or solvolysis using perchloric acid and then the unconjugated metabolite was chromatographed on paper using appropriate paper chromatographic systems.

Final proof of identity and assessment of degree of purity of the isolated metabolites was carried out by isotope dilution as previously described.

AQUEOUS EXTRACT OF ADRENALS FROM INTACT FETUSES

The aqueous soluble material from the adrenal extract weighed 154 mg and contained 220,000 cpm. It was chromatographed on an 100 gm Celite column using a decreasing ratio of iso-octane to t-butanol as the mobile phase and ammonium hydroxide:water (25:475)

as the stationary phase. A plot of the cpm per fraction against the number of fractions is shown in figure 15. On the basis of this diagram certain fractions were pooled and the total radioactivity in cpm was determined. These data, along with any compound isolated from a peak of radioactivity are shown in Table LVI.

The failure to isolate cortisol from the ether soluble extract of the adrenal raised the possibility that this very polar steroid had partitioned into the aqueous phase at the time of the ether:water separation of the initial extract. The elution of a peak of radioactivity from the column at the end of the first hold back volume supported this concept. Paper chromatography of this radioactive material in systems F and E confirmed this and 4700 cpm were mixed with 48.7 mg of authentic non-radioactive cortisol for crystallization. These data along with the results of recrystallization of the acetate formed from the second crystal are shown in Table LVII.

Shortly after the first solvent change two more discrete peaks of radioactive material were eluted. The first of these was tentatively identified as deoxycorticosterone sulphate by paper chromatography in systems H and K. The radioactivity associated with this standard, 7500 cpm, was eluted from the second paper and mixed with 28.6 mg of authentic deoxycorticosterone sulphate that we had synthesized by the method described by Fieser (84). We were unable to find a solvent pair that could yield satisfactory crystals and so the mixture was subjected to solvolysis (83) and the product crystallized following initial purification on an

CELITE COLUMN CHROMATOGRAPHY OF THE AQUEOUS EXTRACT FROM THE ADRENAL OF THE INTACT FETUS

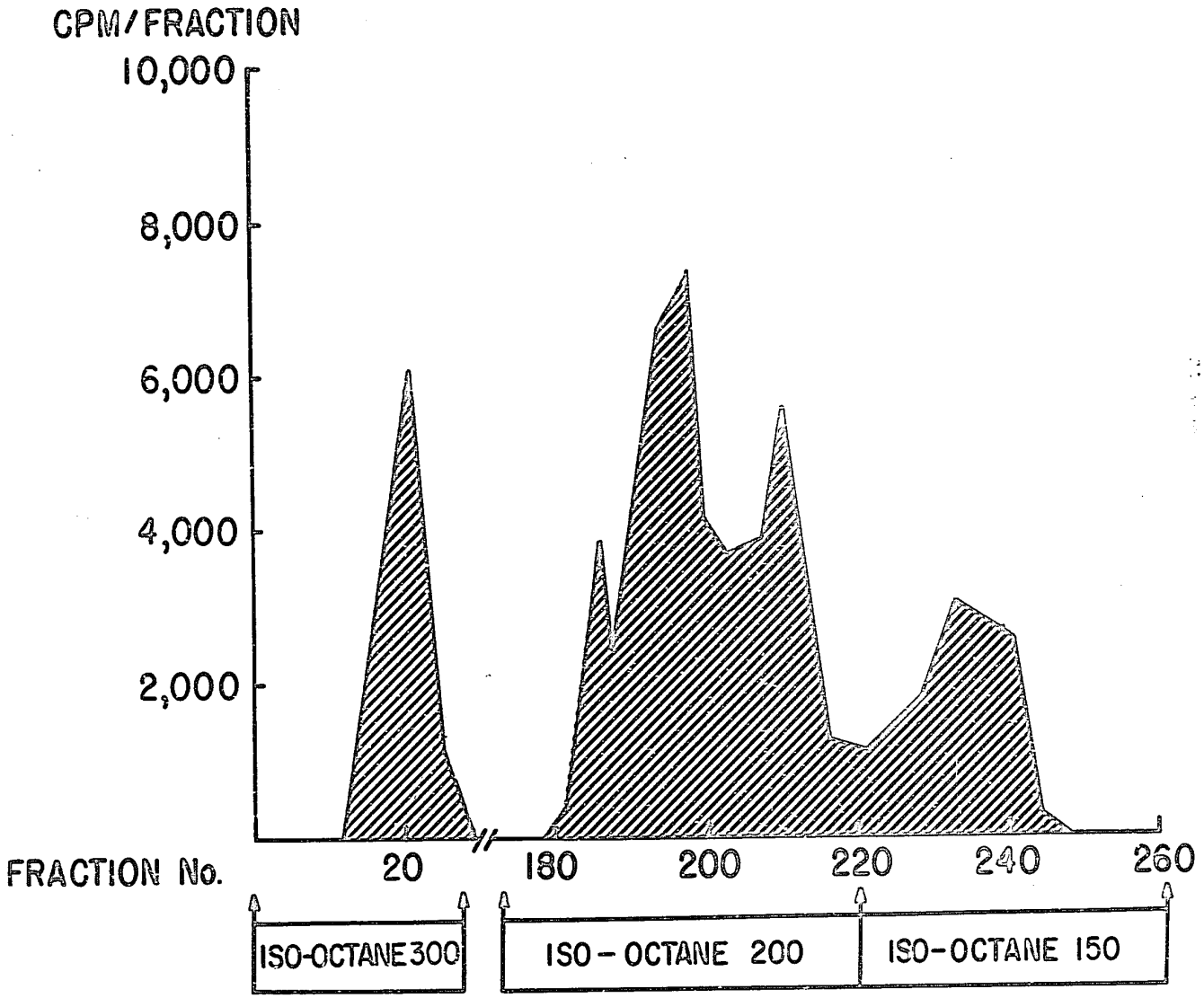

FIGURE 15

TABLE LVI

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE AQUEOUS FRACTION FROM THE ADRENAL EXTRACT

Fractions	cpm	Metabolite Isolated
17-36	44,000	Cortisol, two unknowns
183-187	12,000	Deoxycorticosterone sulphate
188-198	48,000	Corticosterone sulphate
199-213	16,000	Two unknowns
215-232	29,000	Three unknowns
260-267	5,000	Unknown
All others	64,000	Unknown

TABLE LVII

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE AQUEOUS EXTRACT OF THE ADRENAL

(specific activities - cpm/mg)

	Hydrocortisone		Deoxy-corticosterone		Corticosterone Sulphate	
Carrier	48.7 mg		20.0 mg		73.4 mg	
Radioactivity	4700 cpm		5400 cpm		23300 cpm	
Crystallization	XL	ML	XL	ML	XL	ML
1	85	130	210	440	290	360
2	75	160	200	250	280	360
3	65	105	190	200		
4	65	90				
Calculated	95		270		320	

	Hydrocortisone Acetate		Deoxy corticosterone Acetate		Corticosterone	
Crystallization	XL	ML	XL	ML	XL	ML
1	55	55	190	210	310	360
2	55	50	190	200	340	340
3					310	310
Calculated	60		190		380	

	Corticosterone Acetate	
Crystallization	XL	ML
1	320	480
2	320	330
Calculated	350	

alumina column. The material obtained in the third crystallization was then acetylated with acetic anhydride and pyridine and re-crystallized. These data are shown in Table LVII. The final metabolite identified was eluted from the column in fractions 188 to 198. This was chromatographed in systems H and K and 23,200 cpm were eluted from the second paper chromatogram. Having been tentatively identified as corticosterone sulphate on paper, it was mixed with 73.4 mg of authentic carrier and crystallized with great difficulty. The crystalline material was solvolyzed and then crystallized in its free form. As further evidence of proof of homogeneity and degree of purity, the mixture was acetylated and crystallized again. These data are given in Table LVII.

AQUEOUS EXTRACT OF LIVER FROM INTACT FETUSES

This extract was subjected to column partition chromatography on a 100 gm Celite column using the solvent system iso-octane: t-butanol (300:500)/ammonium hydroxide:water (25:475). The polarity of the mobile phase was increased in a discontinuous fashion by decreasing the ratio of iso-octane to t-butanol. The elution of radioactivity from this column is illustrated in figure 16. The times of solvent changes are also shown.

The fractions containing discrete peaks of radioactivity were pooled, and an aliquot taken for counting. The total cpm in each peak and any identified metabolites from the peak is shown in Table LVIII.

CELITE COLUMN CHROMATOGRAPHY OF THE AQUEOUS EXTRACT FROM THE LIVER OF THE INTACT FETUS

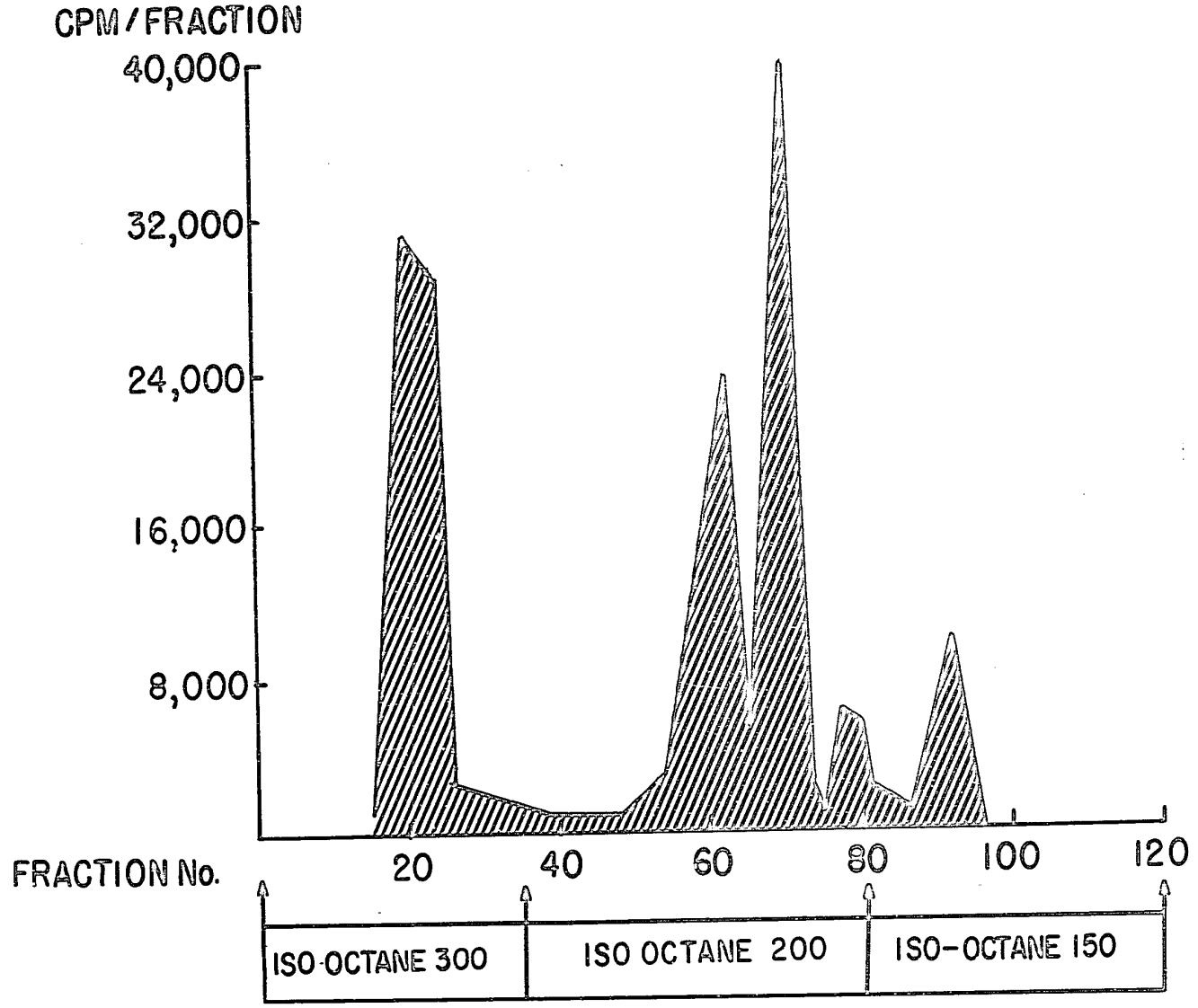

FIGURE 16

TABLE LVIII

TOTAL CPM AND METABOLITES ISOLATED FROM THE RADIOACTIVE PEAKS OBTAINED AFTER COLUMN CHROMATOGRAPHY OF THE AQUEOUS FRACTION OF THE LIVER

Fractions	cpm	Metabolite Isolated
17-27	188,800	Unknown
57-61	43,100	Pregnanolone Sulphate
62-66	95,000	Pregnanediol Sulphate
74-86	38,500	Unknown
87-95	40,000	Pregnanediol Glucuronide
124-152	27,300	Unknown

The initial peak of radioactivity eluted at the end of the first hold back volume was suspected to contain polar unconjugated metabolites but no conjugated or unconjugated compounds were identified.

The radioactive material in the second peak was chromatographed in systems K and J and tentatively identified as pregnanolone sulphate. A total of 34,700 cpm were recovered from the second paper. Approximately one-third of this was taken and solvolyzed and the product was then chromatographed in systems C and A. In each system its mobility was consistent with that of pregnanolone. Following elution from the second paper, 5,700 cpm were mixed with 25.2 mg of authentic carrier and crystallized. The third crystals were reduced with sodium borohydride and the resulting 3α,20β-dihydroxy-5β-pregnane was recrystallized to constant specific activity. These data are shown in Table LIX. The remaining 25,800 cpm were mixed with 31.7 mg of carrier pregnanolone sulphate and crystallized as shown in Table LIX.

The third peak containing 95,000 cpm was chromatographed in systems K and J and 82,000 cpm were eluted from the second paper. Approximately one third of this was solvolyzed and the product was then chromatographed in systems C and D. In each case the unknown had the mobility of pregnanediol. A total of 18,000 cpm was eluted from the second paper and mixed with 71.4 mg of carrier and crystallized. The material from the third crystallization was acetylated and then recrystallized. These data are also shown in Table LIX.

TABLE LIX

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE AQUEOUS EXTRACT OF LIVER FROM INTACT FETUSES

(specific activities - cpm/mg)

	Pregnanolone		Pregnanolone Sulphate		Pregnanediol*		Pregnanediol**	
Carrier	25.2 mg		31.7 mg		71.4 mg		38.4 mg	
Radioactivity	5700 cpm		25800 cpm		18000 cpm		10700 cpm	
Crystallization	XL	ML	XL	ML	XL	ML	XL	ML
1	155	310	780	980	240	385	260	200
2	160	170	615	815	245	290	275	240
3	155	170	410	685	225	200	275	260
4			445	500				
Calculated	225		815		250		270	

	3α,20β-dihydroxy-5β-pregnane		Pregnanediol Diacetate	
	XL	ML	XL	ML
1	165	155	205	200
2	170	155	205	200
3	160	150		
Calculated	155		195	

* - from pregnanediol sulphate ** - from pregnanediol glucuronide

Since the monosulphates of pregnanediol were not available we could not carry this proof further by crystallizing the isolated material as a sulphate.

Despite sequential chromatography and solvolysis no metabolite was identified from the fourth discrete peak of radioactivity.

The radioactive material in the fifth peak was chromatographed initially in system K and the eluate from this chromatogram was divided into two equal portions A and B. Portion A was first subjected to solvolysis and 6000 cpm were dissolved in ethyl acetate. The nature of this radioactive material was not established. The remaining 14,000 cpm which were still aqueous soluble were then hydrolyzed with β-glucuronidase and the resulting product was soluble in ethyl acetate. When this material was chromatographed in system A it had the mobility of pregnanediol. A total of 10,400 cpm were recovered from this paper, mixed with 38.4 mg of non-radioactive pregnanediol and crystallized in this form and as the diacetate as shown in Table LIX. Since the pregnanediol was isolated and identified only after solvolysis and β-glucuronidase hydrolysis had been carried out it was important to prove whether the pregnanediol was present as a glucuronide or as a "sulpho-glucuronide". To prove this, the part B of the eluate from the first chromatogram was subjected to β-glucuronidase hydrolysis first. On this occasion 14,000 cpm were dissolved in ethyl acetate and 6,000 cpm remained soluble in water. The material soluble in

ethyl acetate was tentatively identified as pregnanediol by its mobility in system A. Thus it is apparent that the pregnanediol present in this peak of radioactivity eluted from the Celite column was present as a glucuronide and not as a "sulpho-glucuronide".

No metabolite was isolated from peak 6 by either chromatography or β-glucuronidase hydrolysis.

AQUEOUS EXTRACT OF RESIDUE FROM INTACT FETUSES

This extract weighed 8.2 gm and contained 640,000 cpm. It was chromatographed on a 100 gm Celite column using the system iso-octane: t-butanol (300:500)/ammonium hydroxide:water (25:475). Solvent changes were made as indicated by the appearance of radioactivity from the column. These changes involved decreasing the amount of iso-octane in the mobile phase. The times of these changes are shown in figure 17. This figure shows that only two important peaks of radioactivity were obtained from this column, and the appropriate fractions were combined.

The radioactive material in peak one was chromatographed in systems F and D and its mobility suggested that this was composed of unconjugated material but no identification was achieved.

A total of 230,000 cpm were recovered in the second peak. This material however was quite oily and coloured. The residue weighed 241 mg which made it difficult to proceed directly to paper chromatography. As the next step this material was rechromatographed on a 20 gm Celite column which was set up using the system

CELITE COLUMN CHROMATOGRAPHY OF THE AQUEOUS EXTRACT FROM THE RESIDUE OF THE INTACT FETUS

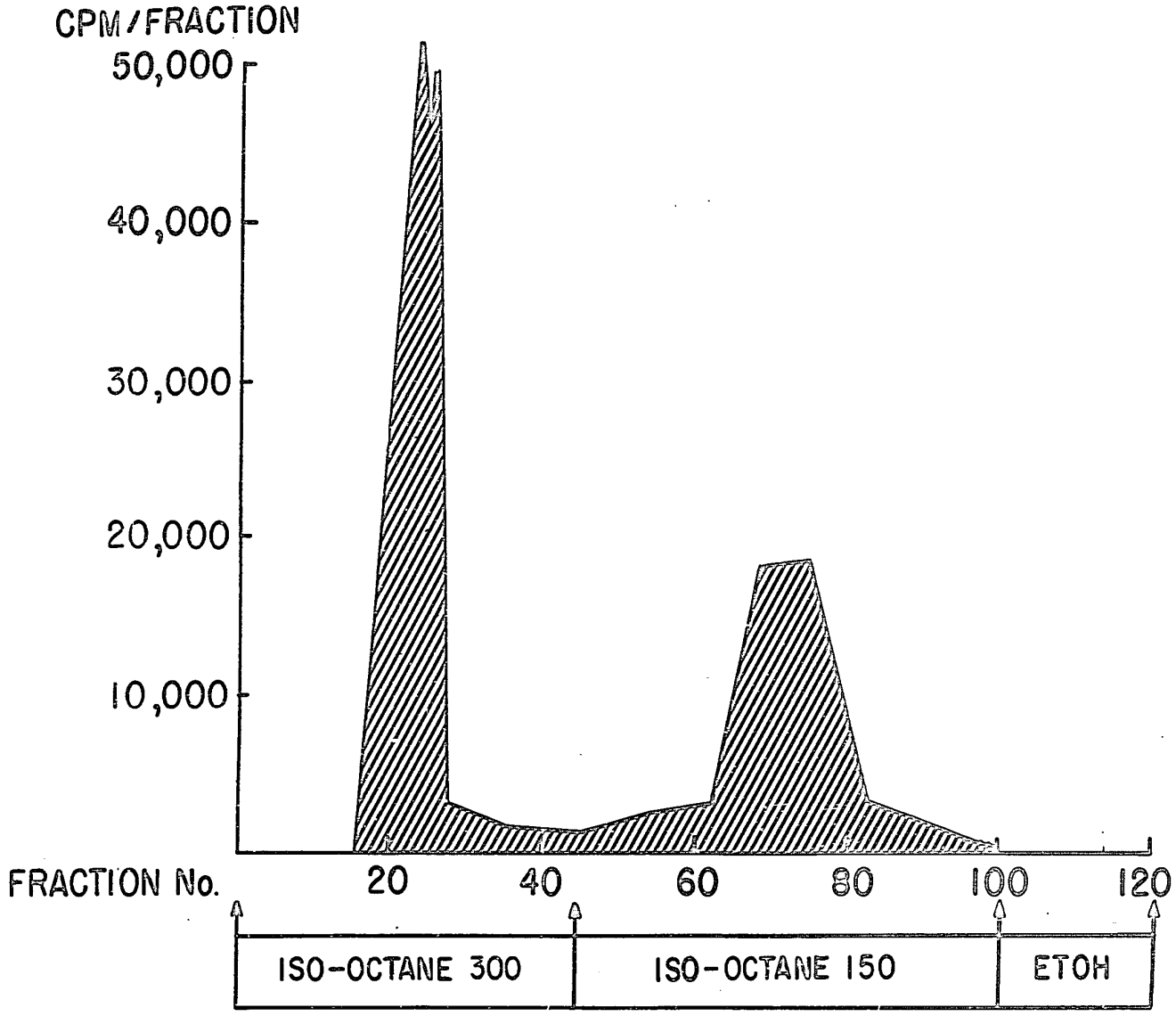

FIGURE 17

iso-octane:t-butanol (200:500)/ammonium hydroxide:water (25:475). The radioactivity from the column was eluted in three poorly defined peaks designated as A, B, and C. This is shown in figure 18.

Peak A, weighed 3.7 mg and contained 22,000 cpm. This was subjected to chromatography and after solvolysis no metabolite was isolated.

Peak B, weighing 55 mg and containing 23,000 cpm was chromatographed in system H and three peaks numbered B_1, B_2, and B_3 were obtained. No identification of a metabolite was made from peak B_1. The material designated B_2 containing 5,500 cpm was rechromatographed in system H, the eluate was solvolyzed and then the product chromatographed in system A where it was identified as 3β-hydroxy-5α-pregnan-20-one. A total of 1,700 cpm was recovered from this paper, mixed with 16.8 mg of carrier and crystallized. After the third crystallization, the crystalline material obtained was reduced with sodium borohydride and the product recrystallized. These data are shown in Table LX. Area B_3 containing 5,600 cpm was rechromatographed in system H, and the single peak was eluted, solvolyzed and chromatographed as an unconjugated metabolite in system A where it was identified as pregnanediol. A total of 2,000 cpm was eluted from this paper, mixed with 16.2 mg of carrier pregnanediol and crystallized. After four crystallizations 5.6 mg of material was obtained which was acetylated and recrystallized. These data are shown in Table LX.

CELITE COLUMN OF PEAK TWO FROM THE INITIAL CHROMATOGRAPHY OF THE AQUEOUS EXTRACT OF THE RESIDUE FROM THE INTACT FETUS

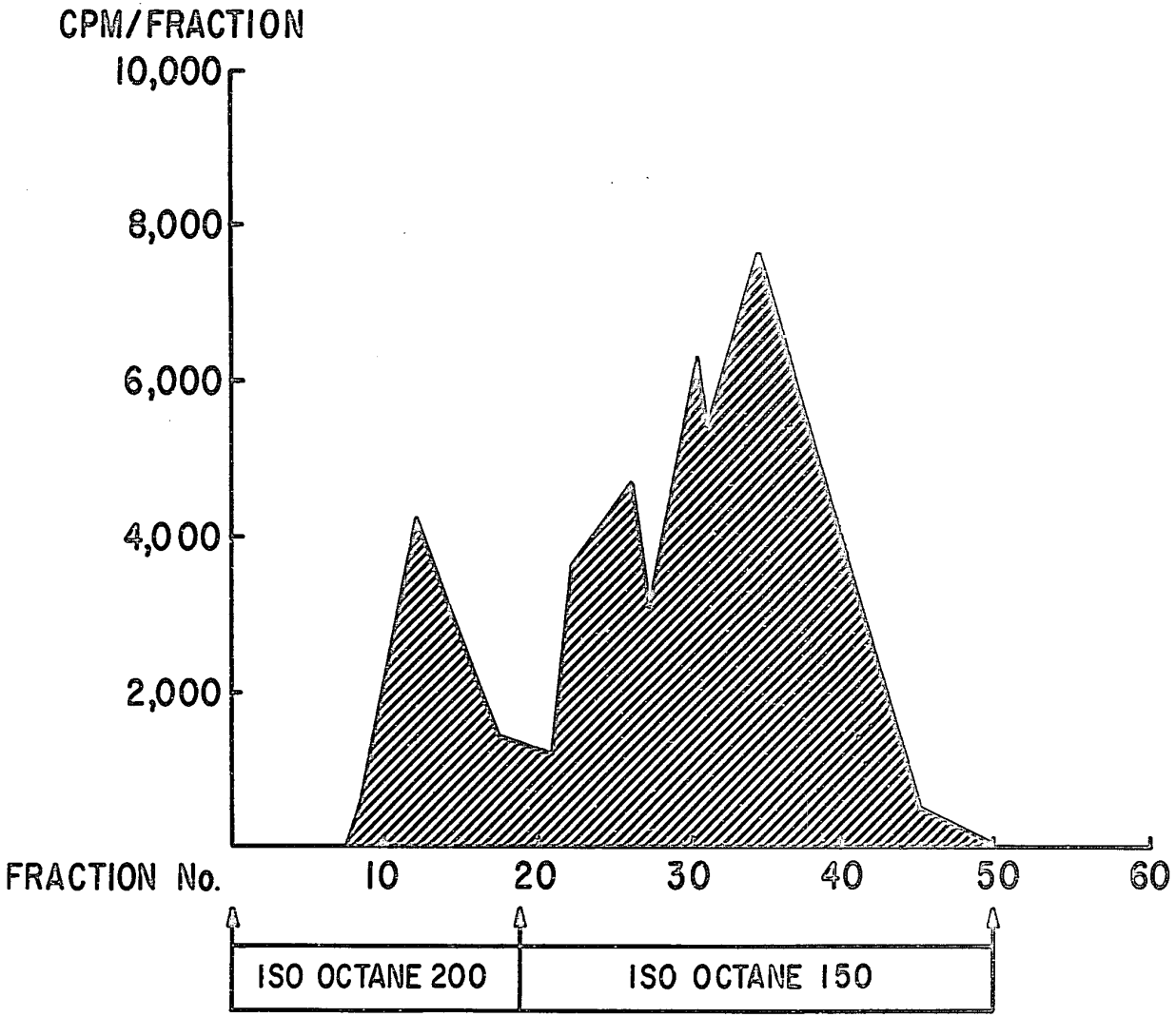

FIGURE 13

TABLE LX

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF METABOLITES ISOLATED FROM THE AQUEOUS EXTRACT OF RESIDUE FROM INTACT FETUSES

(specific activities - cpm/mg)

	3β-hydroxy-5α-pregnan-20-one*		Pregnanediol**	
Crystallization	XL	ML	XL	ML
1	50	165	70	205
2	50	75	65	95
3	50	55	60	75
4			60	60
Calculated	100		130	

	3β,20β-dihydroxy-5α-pregnane		Pregnanediol Diacetate	
	XL	ML	XL	ML
1	60	25	50	45
2	50	50	50	50
Calculated	50		50	

*-from 20-keto-5α-pregnane-3β-yl sulphate

**-from pregnanediol sulphate

Although the radioactive material in area C was chromatographed and then dissolved in ethyl acetate after solvolysis, no metabolite was identified.

ISOLATION OF CONJUGATED METABOLITES FROM THE TISSUES OF THE PERFUSED ADRENALECTOMIZED FETUSES

AQUEOUS EXTRACT OF LIVER FROM ADRENALECTOMIZED FETUSES

Following the ether:water partition of the extract from the liver of the adrenalectomized fetus, 900 mg of residue containing 1.09×10^6 cpm was obtained in the aqueous extract. It was chromatographed on a 100 gm Celite column using the system iso-octane:t-butanol (300:500)/ammonium hydroxide:water (25:475). Aliquots of each fraction were counted for radioactivity and the cpm per fraction were plotted against the fraction number as shown in Figure 19. The more polar radioactive metabolites were eluted from the column by decreasing the concentration of iso-octane in the mobile phase. These solvent changes are shown in Figure 19. Only two metabolites were successfully identified and purified. The first, pregnanediol sulphate was isolated from the second major radioactive peak eluted from the column (fractions 64-83) and the second, pregnanediol glucuronide was isolated from the pool of fractions 109 to 141.

CELITE COLUMN CHROMATOGRAPHY OF THE AQUEOUS EXTRACT FROM THE LIVER OF THE ADRENALECTOMIZED FETUS

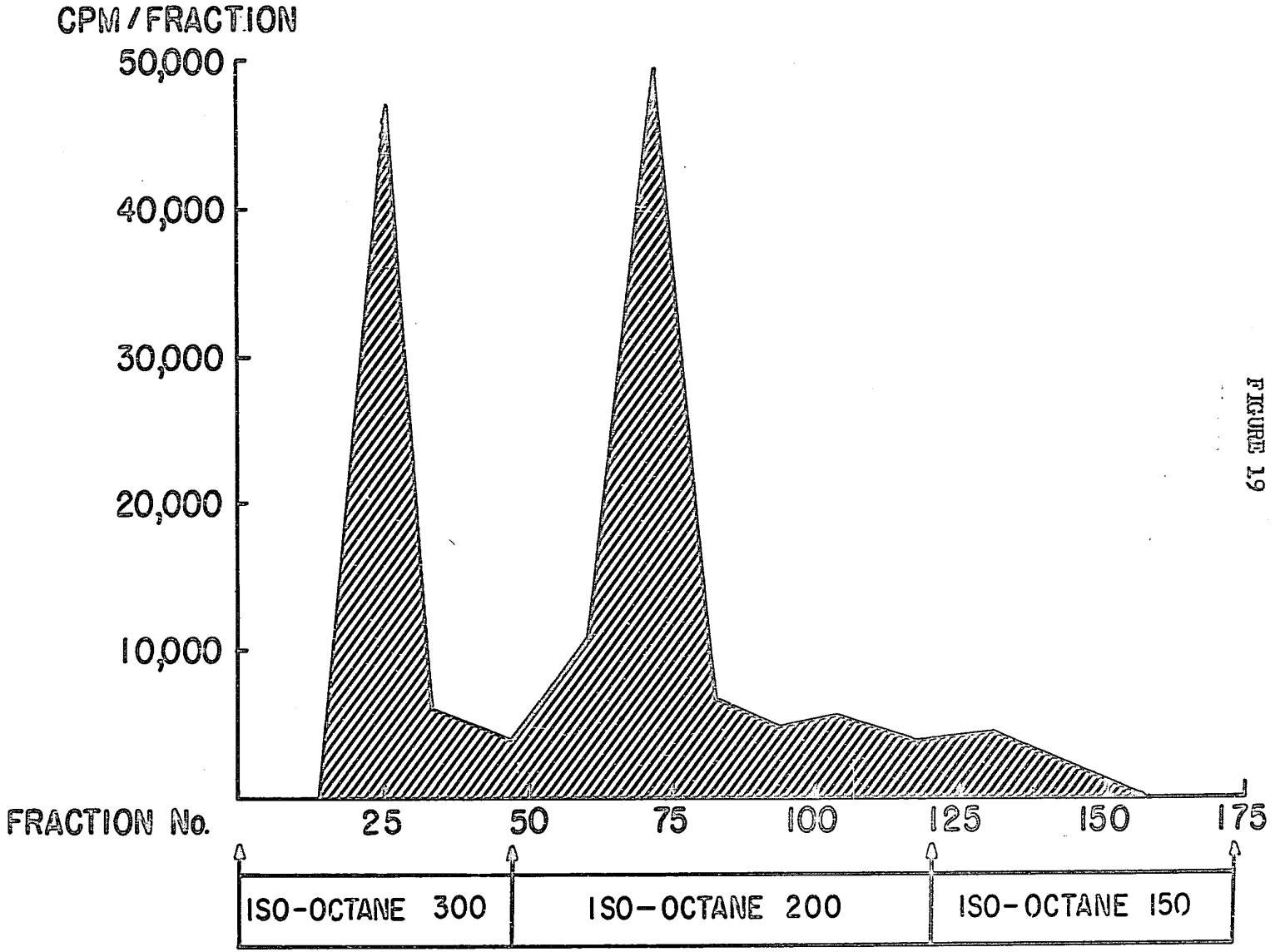

FIGURE 19

The pool of fractions 64-83, from which the pregnanediol sulphate was isolated contained 349,000 cpm. This material was chromatographed as a conjugate in system H and then one-tenth of the eluate was solvolyzed. The product was then chromatographed in systems A and C where it had the same mobility as authentic pregnanediol. A total of 11,500 cpm were recovered from this final chromatogram. One half of this eluate was mixed with 20 mg of carrier pregnanediol and crystallized. The crystalline material obtained after the third crystallization was then acetylated and the resulting diacetate was recrystallized. These data are shown in Table LXI.

A total of 127,000 cpm were obtained when fractions 109-141 were pooled. This material was chromatographed as a conjugate in system K twice and then one half of the eluted metabolite was hydrolyzed using β-glucuronidase. The resulting product which was soluble in ethyl acetate was chromatographed in system C where its mobility was identical with that of pregnanediol. A total of 11,000 cpm from the eluate was mixed with 26.2 mg of authentic pregnanediol and was then crystallized, first as the free steroid and following acetylation as its diacetate. These data are shown in Table LXI.

TABLE LXI

DEMONSTRATION OF RADIOCHEMICAL PURITY OF METABOLITES ISOLATED FROM THE AQUEOUS LIVER EXTRACT FROM THE ADRENALECTOMIZED PERFUSED HUMAN FETUS

(specific activities - cpm/mg)

	Pregnanediol Sulphate	Pregnanediol Glucuronide
	Solvolysis	β-glucuronidase hydrolysis
Carrier	20.0 mg	26.2 mg
	5700 cpm	11000 cpm
	Pregnanediol	Pregnanediol
	XL ML	XL ML
1	255 330	425 405
2	245 270	405 405
3	255 255	
Calculated	290	420
	Pregnanediol Diacetate	Pregnanediol Diacetate
	XL ML	XL ML
1	205 185	340 310
2	195 200	325 320
Calculated	200	320

THE AQUEOUS EXTRACT OF THE RESIDUE FROM ADRENALECTOMIZED FETUS

The residue in the aqueous fraction from the extract of the residual tissues was chromatographed on a 100 gm Celite column using the system iso-octane:t-butanol (300:500)/ammonium hydroxide: water (25:475). By decreasing the amount of iso-octane in the solvent system, the polarity of the solvent was increased. The pattern of radioactivity eluted from the column is shown in Figure 20, where the cpm in each fraction have been plotted against the fraction number. As is shown in the figure, the single major peak was eluted at the end of the first hold back volume. Very polar free steroid metabolites were suspected in this peak but none were identified.

Pregnanediol glucuronide was isolated in the pool of fractions 179 to 195, which contained 107,000 cpm. This material was first chromatographed as a conjugate in system K where it separated into three areas. The third area which contained the pregnanediol glucuronide was chromatographed in system K again. One half of the eluate from this chromatogram was subjected to hydrolysis with β-glucuronidase and then the product was chromatographed in system C. The mobility of the unknown and of pregnanediol were identical in this system. A total of 2,600 cpm eluted from the paper were mixed with 21.2 mg carrier and crystallized in the free form and after acetylation as the diacetate. The results of these crystallizations are shown in Table LXII. The very low specific activity suggests that pregnanediol glucuronide may have been identified.

In all tissues except the adrenal, the great majority of

CELITE COLUMN CHROMATOGRAPHY OF THE AQUEOUS EXTRACT FROM THE RESIDUE OF THE ADRENALECTOMIZED FETUS

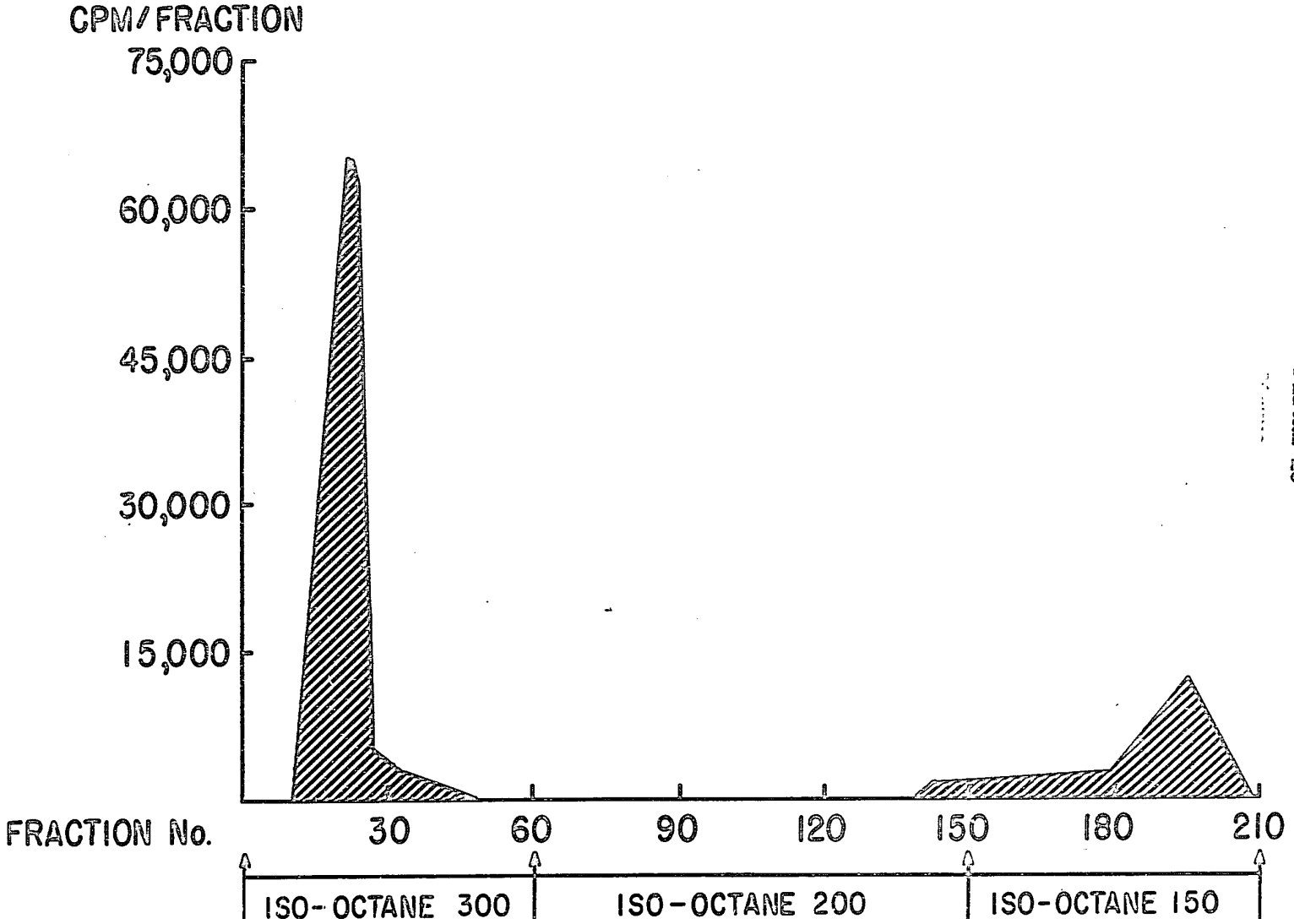

FIGURE 20

TABLE LXII

DEMONSTRATION OF RADIOCHEMICAL HOMOGENEITY OF THE METABOLITE ISOLATED FROM THE AQUEOUS EXTRACT OF THE RESIDUE FROM THE PERFUSED ADRENALECTOMIZED FETUSES

(specific activities - cpm/mg)

Pregnanediol Glucuronide

β-Glucuronidase

21.2 mg carrier

2600 cpm

	Pregnanediol	
	XL	ML
1	24	46
2	25	33
3	24	25
Calculated	125	

	Pregnanediol Diacetate	
	XL	ML
1	18	19
2	19	18
Calculated	19	

radioactivity was found in the ether soluble fraction of the tissue extracts. The yield of metabolites as conjugates from the aqueous fraction was low in most cases as shown in Table LXIII. These data are expressed as the per cent of total radioactivity in a tissue which is found as a specific metabolite. The total activity in a tissue is the sum of the cpm in the aqueous and ether fractions after partition. The radioactivity as a metabolite is the product of the weight of carrier used in the crystallization and the final constant specific activity of the crystals. This is a minimum figure, not corrected for losses. Although all the metabolites shown in Table LXIII were definitely isolated, the minimum significant figure in terms of yield is 0.5 per cent. It is particularly evident when considering the conjugated metabolites, that the per cent yield was very low in most cases.

TABLE LXIII

CONJUGATED METABOLITES OF 4-^{14}C-PROGESTERONE ISOLATED FROM TISSUES OF THE INTACT (INT) AND ADRENALECTOMIZED (ADX) PREVIABLE HUMAN FETUSES (EXPRESSED AS PER CENT OF TOTAL RADIOACTIVITY IN THE TISSUE)

	Adrenal	Liver		Residue	
		INT	ADX	INT	ADX
Corticosterone sulphate	3.9	-	-	-	-
Deoxycorticosterone sulphate	0.7	-	-	-	-
Pregnanolone sulphate	-	< 0.5	-	-	-
20-keto-5α-pregnan-3β-yl sulphate	-	-	-	< 0.5	-
Pregnanediol sulphate	-	< 0.5	<0.5	0.5	-
Pregnanediol glucuronide	-	< 0.5	<0.5	-	0.5

- not isolated.

DISCUSSION

The experiments which have been described were designed to explore in a broad manner, the metabolism of progesterone in a number of fetal tissues at mid-pregnancy. The perfused, previable human fetus in the intact and in the totally adrenalectomized state served as the model system. Normal fetuses were obtained at therapeutic abortion carried out in Sweden at midterm under the control of the Royal Medical Board of Sweden. The perfusion of the fetuses and initial extraction of the tissues were carried out in the laboratoroes of Dr. E. Diczfalusy in the Karolinska Hospital, Stockholm. The separation and isolation of the radioactive metabolites were carried out by myself in the Endocrine laboratories of the Royal Victoria Hospital, Montreal.

In this type of experimental approach the fetus was immersed in a bath under controlled environmental conditions, and oxygenated human blood was perfused through the fetus under the conditions detailed by Westin (68). Under these experimental conditions of controlled temperature and oxygen tension Westin has maintained fetuses for up to four hours and observed a stable electrocardiogram and rhythmic fetal movements for the entire experiment. A successful perfusion for our study lasted one hour during which time the fetal heart continued to beat. During this time, small, equal amounts of 4-^{14}C-progesterone were added to the perfusing fluid at regular, short intervals. At the completion of the one hour perfusion, fetal tissues were extracted and the labelled

metabolites were separated into ether and water soluble portions.

In some cases total bilateral adrenalectomy was performed prior to the perfusion. This was carried out by Dr. R. Wilson working in Dr. Diczfalusy's laboratory in Stockholm.

It is realized that the experimental model was studied under conditions which were quite different from the physiological conditions existing in utero. Considering the variation in results that have been obtained with in vitro incubations of slices or homogenates of fetal tissues (40,41,42) we felt that our experimental design approached the physiological situation more closely. Because the experimental conditions were not perfect, our results have been interpreted with some caution.

At the end of perfusion, the tissues were first extracted with absolute ethanol and then with 80 per cent ethanol and the residues of the ethanol extracts were defatted by dissolving them in 70 per cent methanol and precipitation of the fats at -20°C. The residues of the supernatants were then partitioned between ether and water and the radioactive material from each phase was purified by column chromatography. The extracts of the ether phase were chromatographed on silica gel columns and the extracts from the aqueous phase were chromatographed on a Celite partition column. Individual metabolites or mixtures eluted from the columns were further separated and purified by paper chromatography. The radioactive metabolites were located on paper with a chromatogram scanner and they were partially characterized by their mobility as compared to standards chromato-

graphed at the same time. When a uniform peak of radioactivity was obtained the metabolite was eluted and mixed with carrier. The mixture was crystallized until the specific activity of the crystals and mother liquors were constant. Then a derivative was formed and the crystallization repeated. These rigid criteria for radiochemical homogeneity were applied to every metabolite isolated in these studies.

The data included in Tables XXXIII, LV, and LXIII are based on this rigorous demonstration of radiochemical purity. The total amount of radioactivity present as a specific metabolite is indicated as a percentage of the total radioactivity in the specific tissue. These are minimum percentage figures as there is no correction for any losses during the isolation procedure. Although the precise figure is small in many cases, particularly in connection with the conjugated metabolites, the rigid criteria make them significant.

The results indicate that at midterm the adrenal and liver are the most active tissues in the human fetus in terms of steroid metabolism. Similar results were found with radioautographic studies following perfusion of 4-^{14}C-progesterone in which there was selective concentration of radioactivity in certain tissues, particularly in the adrenal and liver (87). In addition in vitro incubation studies have clearly established the fact that the human fetal adrenal can actively metabolize steroid hormones under selected experimental conditions.

The results obtained from the perfused intact human fetuses indicate that many tissues at mid-pregnancy are capable of carrying

out extensive metabolism of progesterone. In an effort to define the metabolic capability of fetal tissues in the absence of any direct or permissive action of the adrenal, similar perfusion experiments were carried out using totally adrenalectomized midterm human fetuses.

It was possible to isolate 16α-hydroxyprogesterone from the adrenals, perfusate, kidneys and residue of the intact fetus but it was not found in any tissue from the adrenalectomized fetuses. 16α-hydroxyprogesterone has been isolated from incubation studies using adrenal (40) testicular (52) slices in which progesterone was the substrate. In the perfusion experiments the testes were included in the residual fetal tissues and as such, could be the source of the 16α-hydroxyprogesterone in the residue. The absence of this metabolite from all tissues of the adrenalectomized fetus makes this less likely. It may be that the 16α-hydroxyprogesterone was synthesized in the fetal adrenals and then passed via the circulation to these other tissues.

17α-hydroxyprogesterone was isolated from the adrenals, lungs and residue of the intact fetus and from the residue of the adrenalectomized fetus. This metabolite has also been identified in incubation studies using human fetal adrenal (40) and fetal testes (52) with progesterone as the substrate. As 17α-hydroxyprogesterone was isolated from the residue of both intact and totally adrenalectomized fetuses, it suggests that it may have been formed in the fetal testes.

Despite careful investigation Δ^4-androstenedione was not isolated from any tissue of the perfused fetuses. This is in contrast to the results of *in vitro* incubation of fetal adrenals (37) and fetal testes (50) with progesterone. These variations in results emphasize the difference in the experimental conditions between incubation studies and our perfusion experiments.

It was possible to isolate 6β-hydroxyprogesterone from the perfusate, lungs, and kidneys of the intact fetuses and from the perfusate and lungs of the adrenalectomized fetuses. Thus 6β-hydroxylase is found in tissues other than the adrenal.

Corticosterone was isolated only in the perfusate of the intact fetuses but was found as corticosterone sulphate in the adrenal in relatively large amounts. It may be that corticosterone is produced only as the sulphate at this stage of gestation and that cleavage of the sulphate radical is required before corticosterone can leave the adrenal glands.

Hydrocortisone was isolated only from the fetal adrenal and only in the unconjugated form. Recently, however, the presence of hydrocortisone sulphate has been reported following incubation of full term human adrenals with progesterone (47). In addition it has been isolated from the adrenals at midterm following the injection of 4-^{14}C-progesterone into the umbilical vein with the fetus *in utero* (88).

None of the unknowns had the polarity of 18-hydroxyprogesterone, 18-hydroxycorticosterone, 18-hydroxydeoxycorticosterone or aldosterone.

In addition neither 16α,17α-dihydroprogesterone, nor 16α-hydroxy-hydrocortisone was detected.

It was possible to isolate 20α-dihydroprogesterone from all of the tissues studied in the intact and totally adrenalectomized fetuses. This confirms the previous detection, by paper chromatography, of this compound in several tissues of the midterm fetus injected with labelled progesterone (55).

Pregnanediol was isolated from the liver of both the intact and adrenalectomized fetuses. It was the major radioactive metabolite there, accounting for more than 20 per cent of the total radioactivity. It was also isolated from the perfusate of the adrenalectomized fetuses.

Various fetal tissues showed a high degree of enzymic stereospecificity in the reduction of progesterone to the epimeric pregnanolones. The selectivity in this respect was identical in the intact and adrenalectomized fetuses. The 3α,5β-epimer was isolated only from the extracts of liver and intestinal tract, whereas the 3β,5α-epimer was isolated from the lungs, kidneys and residues. As expected, both epimers were present in the perfusate. Progesterone was found in all tissues studied but only 3 per cent of the radioactive material recovered from the liver and 4 per cent of that found in the adrenals was progesterone. This steroid accounted for as much as 10 to 45 per cent of the total radioactive material recovered from other tissues. These results indicate an extensive metabolism of progesterone by the tissues of the previable midterm fetus and that the liver and adrenal are

the major sites for this metabolism.

Some conjugated metabolites were isolated from certain tissues of the intact and adrenalectomized fetuses. Corticosterone sulphate and deoxycorticosterone sulphate were isolated from the adrenals of the intact fetuses. Pregnanolone sulphate was isolated from the liver and residual tissues of the intact fetuses but was not found in any tissues from the adrenalectomized fetuses. The appropriate epimers were isolated such that it was the 3α,5β-epimer of pregnanolone sulphate in the liver and the 3β,5α-epimer in the residue. Previously it was shown (89,90) that the fetal tissues lack a steroid-3-sulphatase so that the failure to isolate pregnanolone sulphate in certain tissues cannot be attributed to cleavage of the sulphate. A more reasonable explanation would be that it was actually present but in such small amounts that it escaped detection.

Pregnanediol was isolated both as a sulphate and as a glucuronide from the liver of the intact and adrenalectomized fetuses. Pregnanediol sulphate was isolated from the residual tissues of the intact fetuses and pregnanediol glucuronide from the residue of the adrenalectomized fetuses. There is, again, no obvious explanation for this apparently selective conjugation of pregnanediol in the residue of the two experimental groups.

A comparison of the results obtained between the intact and adrenalectomized groups shows a remarkable similarity which is further support for the autonomy of the individual tissues

independent of any influence of the adrenal. The relatively high concentration of hydrocortisone (28 µg per ml) added to the perfusing fluid in the adrenalectomized group does not appear to have altered the metabolic fate of the 4-^{14}C-progesterone in the tissues of these fetuses.

As expected no 11β-hydroxylated metabolites of progesterone were detected in the adrenalectomized fetuses and such metabolites were found only in the adrenal or perfusate of the intact fetuses. The failure to isolate any 16α-hydroxylated metabolites in the liver of the intact or adrenalectomized fetuses is of interest as this is in marked contrast to the results obtained with estrone and its sulphate (91,92), when these labelled steroids were perfused through previable human fetuses. When estradiol (89) and dehydroisoandrosterone (93) were perfused, 16α-hydroxylation was demonstrated in the liver again. This suggests further specificity of enzymes involved in the hydroxylation of various steroid substrates in the midterm human fetus.

Of the nine metabolites isolated in these experiments six have previously been reported to be formed from progesterone after incubation with human fetal tissues. Three metabolites, the two epimeric pregnanolones and 6β-hydroxyprogesterone have not previously been reported although 6β-hydroxylated compounds have been found in maternal pregnancy urine (94) and in urine of premature and full term infants (95).

Thus it can be concluded that the tissues of the perfused

previable human fetus can carry out extensive metabolism of progesterone at midterm. This includes hydroxylation, reduction, and conjugation. There is evidence of tissue specificity for many of these reactions.

It is clear that the fetal adrenal in particular attains a phase of active function quite early in the gestational period and that it can produce hormones which in the adult are biologically active. There is evidence in the rat and rabbit (96) that these hormones have specific metabolic effects on the liver and heart of the animal, in terms of glycogen deposition. In humans there is indirect evidence of the production of biologically active steroids by the fetus as manifested by the clinical amelioration of Addison's disease during pregnancy (97).

SUMMARY AND CONCLUSIONS

Previable human fetuses at gestational ages of sixteen to twenty weeks were obtained by abdominal hysterotomy carried out for therapeutic abortion as authorized by the Royal Medical Board of Sweden.

Perfusion with 4-^{14}C-progesterone was carried out under controlled conditions. Two experimental groups were studied. The first, consisting of two male and two female fetuses were intact and the second, consisting of three male and two female fetuses were subjected to total bilateral adrenalectomy prior to the perfusion.

Selected tissues from each experimental group were extracted following a one hour perfusion. The metabolites of 4-^{14}C-progesterone were isolated by column and paper chromatography in the conjugated and unconjugated state. Each metabolite was subjected to crystallization with added authentic carrier to demonstrate radiochemical homogeneity and the degree of purity.

Nine compounds excluding progesterone were isolated, three of which (corticosterone, pregnanolone, pregnanediol) were found both in the form of a conjugate and as the free steroid. One (deoxycorticosterone sulphate) was found only as a conjugate whereas the other five (20α-dihydroprogesterone, 16α-hydroxyprogesterone, 17α-hydroxyprogesterone, 6β-hydroxyprogesterone and cortisol) were isolated only in the unconjugated form.

The results demonstrate that the tissues from the

adrenalectomized group are able to carry out extensive metabolism of progesterone. The results also showed that the 11-hydroxylated metabolites were limited to the intact group as would be expected. Tissues from both experimental groups were able to carry out significant conjugation of metabolites either with sulphuric acid or glucuronic acid. Certain tissues evidenced enzyme specificity in terms of reducing ring A of progesterone to form the epimeric pregnanolones. Tissue enzyme specificity was suggested by the different results obtained when these data were compared to perfusions in which estrone, estradiol, or dehydroisoandrosterone was the substrate.

Definite evidence of the biologic significance of these metabolites produced in the midterm fetus is lacking but the evidence would suggest that the fetal adrenal in particular, is producing compounds which are important in maintaining the fetus in a state of health during this period of rapid growth and development.

CLAIMS TO ORIGINAL RESEARCH

1. The development of chromatographic procedures for the isolation of metabolites from the tissues of the midterm human fetus perfused with progesterone-4-^{14}C.

2. The isolation of corticosterone sulfate, deoxycorticosterone sulfate, pregnanediol sulfate, pregnanediol glucuronide, pregnanolone sulfate and 20-keto-5α-pregnan-3β-yl sulfate, pregnanolone, 3β-hydroxy-5α-pregnan-20-one and 6β-hydroxyprogesterone from human fetal tissues (Biochem. & Biophys. Acta 104: 623, 1965 and J. Clin. Endocr. Metab. 26: 1144 and 1155, 1966).

3. The demonstration of tissue stereo-specificity in the reduction of progesterone-4-^{14}C perfused in the midterm previable human fetus. From the liver and the intestine it was possible to isolate 3α-hydroxy-5β-pregnan-20-one and from the lung, kidney and residue it was possible to isolate 3β-hydroxy-5α-pregnan-20-one.

4. It was demonstrated that tissues other than the adrenals are capable of 17α- and 6β-hydroxylation of progesterone when it was perfused in the adrenalectomized previable midterm fetus.

5. Labeled testosterone, androstenedione, aldosterone and 16,17-dihydroxy steroids were not detected in the fetal tissues which demonstrated a qualitative difference between the findings in the perfused fetus and those obtained from the incubation of progesterone with slices or homogenates of adrenal tissues of the midterm human fetus.

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