11β-HYDROXYSTEROID:NADP OXIDOREDUCTASE IN MOUSE FOETAL TISSUES

by

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ABSTRACT

Corticosterone in foetal tissues after injection of the mother with ^{14}C-corticosterone was determined by acetylation with ^{3}H-acetic anhydride and crystallization to constant specific activity. The corticosterone content of whole foetal tissue varied between gestational days 13 and 17 from 641 to 300 ng/g respectively. The specific activity of foetal hormone recovered remained essentially constant; after a 15-minute pulse this was as much as one-fourth that of maternal hormone. However, placenta, head and liver showed distinctly different patterns of metabolism, which changed greatly during this time in head and liver, with a decrease in the conversion of corticosterone to 11-dehydrocorticosterone and a rise in foetal liver 11β-hydroxysteroid:NADP oxidoreductase activity. This mitochondrial enzyme, Km=33μM, pH optimum 6, which reduces the 11-dehydro metabolite to the biologically active 11β-OH compound, increased sharply, raising the relative amount of the latter in foetal tissues from 15 to 91% during this period. One day after removal of maternal adrenals, foetal corticosterone was normal and maternal levels close to normal, indicating ability of foetal adrenals to function. Maternal hormone, however, crossed to the foetus readily and it was considered most likely that, normally, the maternal source predominates. Regardless of origin, foetal or maternal, however, the hormone is maintained in different foetal tissues in a distinct and different manner.

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LIST OF ABBREVIATIONS

Adrx	:	adrenalectomy, adrenalectomized
AR	:	autoradiogram, autoradiography
CBG	:	corticosteroid binding globulin (Transcortin)
cpd.A	:	11-dehydrocorticosterone
cpd.B	:	corticosterone
DMP	:	2,4,6-tri-(dimethyl)-amino methyl phenol
DDSA	:	dodecyl succinic anhydride
LM	:	light microscopy
L/S	:	lecithin/sphingomyelin ratio
MDA	:	methyl nadic anhydride
PO	:	propylene oxide
RDS	:	respiratory distress syndrome
SEM	:	standard error of the mean
TEM	:	transmission electron microscope
TLC	:	thin layer chromatography
UV	:	ultraviolet

INTRODUCTION

The endocrine control of pregnancy is provided both by the mother and the foeto-placental unit. In most species, the peptide hormones; e.g., ACTH, and serum proteins do not cross the placenta and serve to regulate the levels of steroids on the maternal side. On the other hand, the steroid hormones can cross the placenta. The corticosteroids cross in significant amounts to the foetal side in some species, notably mouse and man. Oestrogens and androgens, as well as the catecholamines and thyronines are subject to a very rigourous metabolic control, mainly by sulfurylation in foetal tissues (1). Whereas in the placenta oestrogens and other steroids hormones are present predominantly in an unconjugated "free" form, they are extensively and rapidly conjugated in the foetus.

One of the important factors which is thought to influence the growth and secretory activity of the foetal adrenal is the secretion on ACTH by the foetal pituitary. A review by Jost (2) summarized experimental evidence on that aspect in the mouse, rat, sheep, rabbit and guinea pig. Absence of the pituitary, destruction of the heads by X-ray beams or surgical decapitation of foetuses all result in a decrease in size of the adrenal cortex at birth, atrophy of the cortical cells and modification in the distribution of lipids, which can be reversed by giving ACTH to the foetuses at the time of surgery.

In the human anencoephalic foetus, the foetal zone of the foetal adrenal regresses prematurely and the definitive cortex, while hypoplastic, undergoes premature differentiation. Hence it seems reasonable to believe that ACTH deficiency is responsible for the adrenal abnormalities in the anencoephalic infant.

Administration of the synthetic corticosteroid, dexamethasone, to pregnant Rhesus monkeys (3) resulted in atrophy of foetal adrenal gland and an appreciable regression of the foetal zone. Dexamethasone is believed to suppress pituitary ACTH selectively. If this assumption can be correctly applied to the foetus, these experiments provide strong support for the belief that ACTH is an important trophic factor for the foetal zone of the adrenal in utero.

That the foetus can synthesize corticosteroids is well established. Dupouy et al (4, 5) have shown in the rat following adrenalectomy on the mother on day 18, that the level of corticosterone in maternal blood was the same as in the untreated, indicating that not only the foetal rat adrenal has the ability to synthesize corticosteroids, but also that the hormones can cross the placenta from the foetus to the mother, in contrast to the sheep (6, 7).

The levels of corticosteroids are also regulated on the maternal side by transcortin. This protein, also called

corticosteroid binding globulin (CBG), binds specifically cortisol, corticosterone, their 11-deoxy metabolites and progesterone, but not any of their other metabolites (8). It is present in most species examined and is synthesized in the liver. The only factors that seem to influence the levels of transcortin are pregnancy and the level of oestrogens. Only about 10% of the total corticosteroid is free, the rest being bound to transcortin.

Gala and Westphal (9) have reported a marked increase during pregnancy in the corticosteroid-binding activity in the serum of the mouse, rabbit and guinea pig, followed by a decline to the level of non-pregnant animals during lactation. Free serum corticosteroid concentrations paralleled the bound steroid but were only a fraction of the total.

This increase occurs also in human and other species, the level of total corticosteroid increasing severalfold, the free steroids increasing proportionately. The transcortin-bound steroid is not able to enter tissues, and does not cross the placenta and biological activity is attributed only to the free steroid (8).

The passage of corticosteroids across the placenta shows a pattern which varies with species and which is at least partially determined by the type of placenta. Liggins (6) has shown in the ewe that corticosteroids cross the placenta

in the direction foetus to mother, but not in the reverse direction. On the other hand, adrenalectomy of rats (4) and in mice (2) has shown that, when the maternal source of steroid synthesis is removed, the maternal steroid levels are restored to normal within a few hours by synthesis in foetal adrenals. Measurements of cord blood cortisol in the human (10, 11, 12, 13) suggest that corticosteroids also cross the placenta in both directions. Comparison of the placenta involved (14) shows that the sheeps possess the syndesmochorial type, which is characterized by chorionic villi occupying deep pits in the uterine lining, with some local destruction of the uterine epithelium which allows the chorionic ectoderm to come into direct contact with the vascular maternal connective tissue. The rodents and the human possess a haemochorial type of placenta which is characterized by a more thorough erosion of the superficial uterine mucosa and the branching chorionic villi are directly bathed by maternal blood issuing from opened vessels.

The active steroid is also metabolized in the foeto-placental unit. An enzyme, 11β-hydroxysteroid:NADP oxidoreductase has been identified in mouse and in human foetal tissues (15, 16). This enzyme dehydrogenates the 11β-hydroxyl group which is essential for biological activity of the hormone, forming 11-keto metabolites. This is considered to be responsible for the high levels of cortisone in human cord blood (17). Burton et al (18) noted in the mouse an

inverse relationship between the rate of conversion of corticosterone to 11-dehydrocorticosterone in foetal tissues and the capacity of corticosterone to elicit an increase in liver glycogen. A sharp increase in glycogen deposition occurred in foetal liver paralleling a decrease in the rate of conversion of ^{14}C-corticosterone to ^{14}C-11-dehydrocorticosterone. This was thought to be due, not to a decrease in dehydrogenase activity, but rather to the appearance of an enzyme in foetal liver which reduced the metabolite back to corticosterone. This activity developed only late in gestation and resulted in a rise in corticosterone at the expense of the metabolite.

Corticosteroids have been shown to play major roles in foetal development. Experimental work has shown that functional maturation of foetal lamb lungs can be accelerated by stimulation of the foetal adrenal cortex or by administration of glucocorticoids (6). Liggins suggested that glucocorticoids caused liberation of surfactant into the alveoli, perhaps by induction of an enzyme concerned with the biosynthesis of surfactant.

Gluck (19) has devised a biochemical method to assess the maturity of the foetal lung. After having presented evidence that there is continuity between the foetal lung and amniotic fluid, it was suggested that the amniotic fluid lecithin/sphingomyelin (L/S) ratio would reflect the degree

of foetal lung maturity. It has been shown that corticosteroids induce the enzyme choline phosphotransferase, increasing the synthesis of phosphatidyl choline (20), which is the critical factor in the surfactant which lowers surface tension in the alveoli.

Investigations by Murphy on human infants (21) have revealed that umbilical cord blood cortisol and cortisone levels were lower in infants with respiratory distress syndrome (RDS) than in the normal controls. Also, in the case of premature rupture of membrane, a low cortisol level was accompanied by RDS, whereas a higher cortisol level was not. The cortisol level was always at least double in those infants without RDS. These findings provide a physiologic justification for attempted prevention of disease resulting from pulmonary immaturity in the human by the prenatal administration of glucocorticoids. Controlled trials of betamethasone therapy have been carried out (22) in mothers in whom premature delivery was threatened. The respiratory distress syndrome occurred less often in treated babies and treatment has become widely applied in the last two years.

The importance of the functional foetal hypothalamus-pituitary-adrenal axis on the onset of labour has been demonstrated in the sheep (7). Liggins has shown that infusion of adrenocorticotropic hormone (ACTH) or cortisol into the foetal lambs induced premature parturition, whereas

infusion of these hormones into the ewes did not. In the same animal, Drost and Holm (23) have shown that pregnancy can be prolonged by adrenalectomy of the foetal lamb. In the rabbit, if adrenal corticosteroids play a role in initiating labour, Nathanielsz and Abel (24) did not succeed in showing whether they are secreted by the maternal or the foetal adrenals. However, in the foetal rat, the plasma corticosterone concentration was shown to be as high as six times maternal values at day 19 of gestation (gestation period: 22 days) and to be approximately equal to maternal values from day 20 to term (25).

The direct participation in the onset of labour of the foetus has not been proven in the human but some evidence suggests it; e.g., the prolongation of pregnancy associated with anencoephaly (the absence of pituitary formation (26)). Cortisol levels are three times higher in foetus at 32 weeks than at 18 weeks (10). However, premature infants with RDS sometimes have very low cortisol levels, suggesting that while cortisol is essential for normal development, it is not the primary factor initiating labour, at least when this occurs prematurely. Injection of dexamethasone into Rhesus monkeys did not induce parturition (3). So, possibly in primates, the corticosteroids are not the primary factor inducing parturition.

Because of the similarity of the placenta of the human and the mouse (14), which is of the haemochorial type, it was reasoned that the mouse provides a model for steroid transport and metabolism in mammals in those instances where the hormone has been shown to cross the placenta easily and is metabolized extensively to the 11-keto derivative.

Since there is already a pool of steroid in the animal at the moment of the experiment, the administration of radioactive hormone results in a dilution of the isotope, the extent of which can be used to estimate changes in the relative size of the pool.

To determine accurately small amounts of corticosteroids in the maternal blood and/or in the foetal tissues, several methods have been used. Murphy (28) has developed a competitive protein-binding radio-assay. Burton (16) used a fluorometric method which has the limitation of detecting only the active form of the hormone and of requiring larger volumes of blood. Values obtained agree well with other methods (28, 29). Although reducing enzymatically the inactive metabolite to corticosterone made it possible to measure the former fluorometrically, it would be difficult to use with the mouse because of the small quantities of blood and tissue.

An even more sensitive method has been developed by Hillman and Giroud (29) whereby a trace amount of ^{14}C-labelled hormone is acetylated with acetic-^{3}H anhydride and purified by several steps of paper chromatography to a constant ratio of $^{3}H/^{14}C$. The method used in this work is a modification in which after acetylation radioinert steroid acetate was added and the mixture was cocrystallized. Constancy of isotope ratio $^{3}H/^{14}C$ after the third and fourth crystallization was accepted as the criterion of purity of the compound.

MATERIALS

1. Animals

UBC Swiss mice were obtained from the zoology vivarium at this university. The gestation period was 19 days. Day 1 was defined as the day following the mating night. To determine the day of pregnancy accurately, the males were left with the females from 9 p.m. to 9 a.m.

2. Buffers

Krebs-Eggleston phosphate buffer contains: 0.9% NaCl, 1.15% KCl, 3.82% $MgSO_4.7\ H_2O$, 0.1M phosphate buffer, pH 7.4 (30).

Phosphate and acetate buffers from pH 4 to 9 were prepared following the procedure of Hawk, Oser and Summerson (31).

3. Solvents

Every solvent utilized was distilled: chloroform, carbon tetrachloride, methanol, ethanol, n-hexane, ethyl acetate, toluene, dichloromethane and acetone.

4. TLC

Only Eastman Silica gel TLC plates with fluorescent indicator were used. They were washed with distilled methanol prior to use.

5. Radioactive compounds

Acetic-^{3}H anhydride (specific activity 400 Ci/mmole) comes in sealed ampules to be protected against humidity. It was purchased in batches of 100 mCi. from New England Nuclear Corp. It was diluted 10 times with non-radioactive acetic anhydride. Anhydrous benzene was then added to give a final concentration of 10% acetic anhydride in dry benzene (v/v). The reagent was stored at room temperature in a desiccator containing DRIERITE.

1, 2-^{3}H corticosterone (specific activity 51.6 Ci/mmole) and 4-^{14}C-corticosterone (specific activity 57.3 mCi/mmole) were purchased from New England Nuclear Corp. Each was spotted onto TLC to purify and was eluted with acetone. ^{14}C-11-dehydrocorticosterone was synthesized enzymatically from ^{14}C-corticosterone (cf. Methods).

6. Non-radioactive reagents

The coenzymes NAD, NADP, NADH and NADPH were bought from Sigma Chemical Company, St. Louis, Mo. Corticosterone, 11-dehydrocorticosterone and corticosterone acetate were obtained from Steraloids, Pawling, N.Y.

7. Scintillation solvent

The scintillation mixture was made of 4 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP) dissolved in 1 litre of toluene.

8. Liquid scintillation spectrometer

The liquid scintillation spectrometer was a Unilux IIA, Nuclear Chicago. The raw data were reduced with a Hewlett-Packard 65 calculator.

9. Transmission electron microscopy

(a) Phosphate buffer

Buffer A: 0.1M sodium cacodylate and 0.5% $CaCl_2$ in distilled water

Buffer B: to every four parts of buffer A, one part of 3.5% NaCl.

For pH 7.2, 143 ml of buffer A and 57 ml of buffer B.

(b) Epon 812

The embedding resin consisted of 28.62 g Epon 812, 21.0 g DDSA, 10.53 g MNA and 1% DMP 30.

(c) Sectioning

Sectioning was performed with a manual ultramicrotome Sorvall MT-I "Porter-Blum".

10. Autoradiography

Medical X-ray films (NS2T) were obtained from Kodak.

METHODS

1. Animals

(a) Injection of mice

Pregnant mice were injected subcutaneously at the back of the neck with 0.9% NaCl containing 0.5 µCi of ^{14}C-corticosterone. Unless otherwise stated, they were sacrificed 15 min. later with an overdose of CO_2, the uterus removed from the mother and kept on ice in Petri dishes containing a filter paper wet with saline. Then the blood was drawn from the mother with a heparinized syringe to prevent coagulation. The foetuses and placentae were removed from the uterus - usually three were pooled - weighed and ground in 5 ml of saline.

(b) Adrenalectomy

Pregnant mice on day 16 were anaesthetized with 0.1 ml/10 g body weight of a solution containing 6.7% nembutal and 0.9% ethanol. The adrenals were removed and the mice were kept under normal conditions with water and food for the next 24 hrs.

2. Tissues

(a) Minced tissues

Foetal tissues were pooled (three heads, three carcasses, three livers), minced on a wet filter paper, put into 1 ml incubation mixture and kept on ice until the time of incubation.

(b) Cell fractionation

The livers from 18-day foetuses from two or three mothers were pooled, homogenized in 6 ml Krebs buffer containing 0.25M sucrose and cell fractionation was performed as follows: After removing 1 ml, the whole homogenate was centrifuged at 1,200 x g for 10 min. The nuclear sediment was homogenized a second time to break the unbroken cells and was re-centrifuged. The mitochondria were centrifuged at 15,000 x g for 20 min and the microsomes at 100,000 x g for 30 min. Each fraction was resuspended in the same buffer and washed twice. The final volume was made up to the equivalent of 50 mg tissue/ml buffer.

3. Incubations

(a) Enzymatic synthesis of 11-dehydrocorticosterone

Guinea pig liver microsomes were obtained by cell fractionation as described for mouse foetal livers and were diluted to the equivalent of 100 mg tissue/ml buffer. They could be stored frozen without significant loss of activity. The incubation mixture consisted of 0.1 ml microsome preparation, 0.5 µCi ^{14}C-corticosterone, 0.1 ml Krebs-sucrose buffer, 1 mg NADP in a 25-ml erlenmeyer flask. The incubation was carried out at 37°C for 30 min. and the flasks were shaken at 120 cycles/min. (16).

(b) Minced tissues

Minced tissues were incubated with 1 ml 0.9% saline or Krebs buffer containing 20,000 cpm ^{3}H-corticosterone or 2,000 cpm ^{14}C-11-dehydrocorticosterone. In order to make possible the development of autoradiograms, the brain tissues were incubated with 10,000 cpm ^{14}C-corticosterone. Unless otherwise stated, the incubations were carried at 37°C in 25-ml erlenmeyer flasks, stoppered with a silicone stopper and shaken at 120 cycles/min. for 15 min. The reaction was

stopped by putting the flasks on ice, followed by extraction with solvents.

(c) Coenzyme specificity

One ml from each fraction was incubated with 0.5 mg coenzyme (NADH or NADPH) and 2,000 cpm ^{14}C-11-dehydrocorticosterone.

(d) pH optimum

Incubations were carried out in 1 ml buffer at different pHs (4 to 9) and 2,000 cpm ^{14}C-11-dehydrocorticosterone. The reduction of 11-dehydrocorticosterone was carried out in flasks gassed with N_2.

(e) Kinetic analysis

The oxidation reaction was carried out with various substrate concentrations, 2,000 cpm ^{14}C-corticosterone, 0.5 mg NADP at pH 8.0. The reduction reaction was carried out with ^{14}C-11-dehydrocorticosterone, 0.5 mg NADPH at pH 5.5.

4. Extraction of tissues

(a) Blood and tissues from mice

Samples were extracted in 6 vol. of n-hexane by shaking vigourously 20 times. The upper phase, containing much neutral lipid, was discarded. Steroids were extracted by addition of 6 vol. of dichloromathane (CH_2Cl_2), shaking 20 times vigourously. CH_2Cl_2 was evaporated down to dryness at 40°C under N_2 gas to avoid oxidation of the steroids. Before drying CH_2Cl_2 down, 10 µg of carrier steroids were added to each tube, one-fifth of each sample was removed for spotting onto TLC to determine the amount of ^{14}C-steroid before acetylation with tritium. The remaining four-fifths were processed through to crystallization.

(b) Incubated minced tissues and cell fractions

In these cases, the entire CH_2Cl_2 fraction was spotted onto TLC.

5. Chromatography

10 µg of the non-radioactive compounds to be detected (corticosterone and 11-dehydrocorticosterone,

or corticosterone and corticosterone acetate) were added to each sample as a carrier for location on the TLC. After evaporation, the extracts were dissolved in two to three drops of chloroform-methanol (3:1) and spotted onto Eastman TLC plates at room temperature in two different systems:

(a) n-hexane: ethyl acetate (4:1) to remove residual fats;

(b) toluene: chloroform: methanol: water (120:60:20:1) to separate steroids.

The zones were located under shortwave UV, cut out and eluted with acetone.

6. Acetylation

Following the procedure of Hillman and Giroud (29) and Kliman (32), 1 vol. of acetic-^{3}H anhydride and 9 vol. of radioinert acetic anhydride were mixed and made to 12% acetic anhydride in benzene, with specific activity of 40 Ci/mmole. Thirty µl acetic-^{3}H anhydride and 20 µl dry pyridine, to keep the reaction mixture in solution, were added to each sample after drying.

The acetic-^{3}H anhydride and pyridine were measured from microvolumetric pipettes stored in a desiccator over DRIERITE until ready for use. After thorough mixing by gentle shaking, the tubes were stoppered VERY tightly and incubated for 24 hrs. at 37°C (33).

0.5 ml 25% ethanol was added to hydrolyze the excess acetic-^{3}H anhydride to acetic acid. The samples were then extracted with 6 vol. CCl_4 by shaking 20 times vigourously to separate the steroids. The water layer was removed and the sample was washed with another 0.5 ml of water to get rid of residual ^{3}H-acetate, which was discarded.

7. Crystallization

After evaporation of CCl_4, the samples containing radioactive corticosterone acetate were dissolved in 1 ml of methanol containing 10 mg of non-radioactive corticosterone acetate. Cold distilled water was added dropwise to the cold samples to allow crystallization to begin. After four to six hours at 4°C, the crystals were redissolved in 1 ml of methanol. One-fifth of each sample was removed after the third crystallization and counted. This procedure was repeated three to four times or until a constant $^{3}H/^{14}C$ ratio was obtained (Table I).

TABLE I. DETERMINATION OF THE PURITY
OF CRYSTALLIZED ^{14}C-CORTICOSTERONE - 21-^{3}H-ACETATE

Sample 1: Mother 3 at day 14

	Crystallization 3	4	5
^{3}H	11,625	9,943	
^{14}C	671	573	
$^{3}H/^{14}C$	17.33	17.35	

Sample 2: Foetus 32 at day 18

^{3}H	7,827	3,696	2,784
^{14}C	212	128	103
$^{3}H/^{14}C$	36.95	28.93	27.08

A difference smaller than 10% between isotope ratio $^{3}H/^{14}C$ after two successive crystallizations was accepted as the criterion of purity of the compound (29).

8. Standards for efficiency of acetylation

Approximately 10,000 dpm of ^{14}C-corticosterone was acetylated at the same time as the samples. The corticosterone zone was cut out of the TLC, eluted with acetone and counted to determine the amount of cpd.B that had not been acetylated. The corticosterone acetate zone was also cut out and eluted. Before proceeding to the crystallization of the latter, one-fifth of each standard was counted; the four-fifths remaining were crystallized.

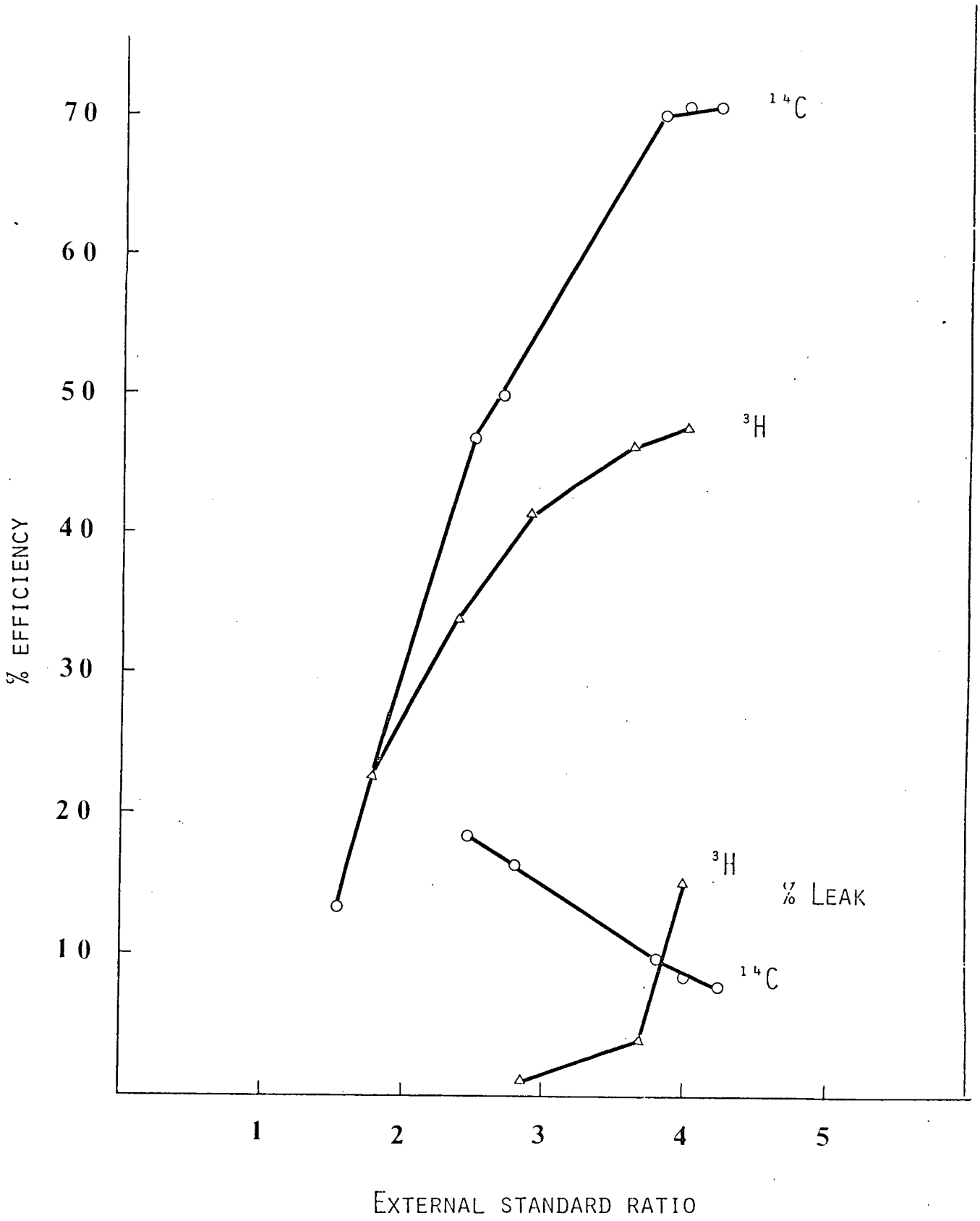
9. Quench curve and calculation of the double label

A quench curve for the determination of the efficiency of counting for ^{3}H and ^{14}C is shown in Figure 1. It was checked at least every other month. The calculations for the double isotope analysis were done according to the method of Kobayashi and Maudsley (34).

10. Transmission electron microscopy

Fixation was performed in glutaraldehyde:phosphate buffer (4:1) for 1 h. The tissues were quickly transferred into a rinse phosphate buffer for 15 min.

FIGURE 1. QUENCH CURVE FOR ^{3}H AND ^{14}C



Post-osmification was done in an osmium tetraoxide: phosphate buffer (1:1) for 40 min. Afterwards the tissues were kept in phosphate buffer until dehydration.

Methanol was used for dehydration because it is the best known to reduce changes in volume of the tissue. It has also smaller molecular size and can penetrate the tissue more easily. The tissues were left for 5 min. in 30%, 50%, 70% and 90% methanol, then 2 x 30 min. in propylene oxide (PO).

The tissues were dehydrated in PO:Epon 812 3:1, 1:1, and 1:3 for 1 h. each. They were embedded in 100% Epon 812 and the resin was allowed to harden at 60^{o}C for 24 h.

The thin sections were stained with 0.2% lead citrate for 20 seconds. Electron micrographs were made at original magnifications of 3,000 to 12,000 with a Philips P-75 electron microscope.

EXPERIMENTAL RESULTS

A. In vivo experiments

1. Corticosterone content of tissues

The total amount of corticosterone in the mother and in the foetuses on days 13 and 17 are shown in Table II. Between these two days, the level of corticosterone increased in the mother but decreased in the foetuses. While the total amount decreased on the foetal side (Table II) ($p<.01$), its specific activity actually increased (Table III), indicating no change in the relative rate of transfer across the placenta. On day 17 the specific activity of foetal corticosterone was about one-fourth that in maternal blood.

TABLE II. TOTAL AMOUNT OF CORTICOSTERONE IN THE MOTHER AND FOETUSES ON GESTATIONAL DAYS 13 AND 17

Gestational Day	Mother ng cpd.B/ml blood ± SEM	Foetuses ng cpd.B/g tissue ± SEM
13	720.3 ±139.3 (5)*	641.9 ±185.4 (3)
17	1,145.4 ±103.5 (6)	300.9 ±48.6 (3)

* number of observations in parentheses

TABLE III. SPECIFIC ACTIVITY OF RECOVERED ^{14}C-CORTICOSTERONE

Gestational Day	Mother # ^{14}C-dpm/ng cpd.B	Foetuses # ^{14}C-dpm/ng cpd.B
13	24.52 ±3.6	0.99 ±0.22
17	12.04 ±1.77	3.44 ±0.47

2. Effect of adrenalectomy of the mother on the total amount of corticosterone

The effect of adrenalectomy of the mother on day 16 on the amount of corticosterone in the mother 24 hrs. later is shown in Table IV. The maternal source of steroid synthesis has been removed, yet the mother's blood contained approximately normal level of hormone (Table IV). The foetal levels of corticosterone were also essentially normal (Table V). This indicates that, in the absence of maternal hormone, the foetal adrenal is capable of functioning and of maintaining substantial production of hormone.

TABLE IV. EFFECT OF ADRENALECTOMY OF THE MOTHER ON THE LEVELS OF CORTICOSTERONE IN THE MOTHER

Sample	ng cpd.B/g tissue ±SEM	^{14}C-dpm/ng cpd.B ±SEM
Controls (6)	1,145.4 ±103.5	12.04 ±1.77
Adrx (2)	1,586.0 312.3	14.92 31.60

TABLE V. CONTENT AND SPECIFIC ACTIVITY OF RECOVERED ^{14}C-CORTICOSTERONE IN THE FOETUS AFTER ADRENALECTOMY OF THE MOTHER

Sample	ng cpd.B/g tissue ± SEM	^{14}C-dpm/ng cpd.B ± SEM
Controls	300.9 ±48.6	3.44 ±0.47
Adrx	437.2	0.58
	1,344.2	0.23
	181.4	2.64
	219.4	2.02

3. Metabolism of ^{14}C-corticosterone in the placenta and the foetal tissues

Table VI shows the relative amount of corticosterone and 11-dehydrocorticosterone in the placenta and foetal tissues recovered in the two tissues 15 min. after injection of ^{14}C-corticosterone into the mother. The accumulation in the placenta of more than 75% of the total counts recovered was constant for days 12 and 14. Most of it was in the form of corticosterone. In contrast, the foetal tissues contained less than 20% of the radioactivity, and most of it was in the form of 11-dehydrocorticosterone, showing an extensive metabolism of the active hormone in the foetal tissues.

TABLE VI. RELATIVE AMOUNT OF ^{14}C-CORTICOSTERONE AND ITS 11-DEHYDROMETABOLITE IN THE PLACENTA AND FOETAL TISSUES

	% of Total Counts Recovered ± SEM			
	Placenta		Foetuses	
Gestational Day	cpd.B	cpd.A	cpd.B	cpd.A
12 (7)	75.6 ±1.5	12.4 ±1.8	1.9 ±0.2	10.2 ±0.5
14 (7)	76.0 ±1.1	3.1 ±0.5	3.5 ±0.2	16.6 ±1.3
16 (8)	55.1 ±1.9	1.4 ±0.2	21.7 ±1.0	22.8 ±1.3

4. Corticosterone and its 11-dehydro metabolite in the foetal tissues

The conversion of corticosterone to its 11-dehydro metabolite was determined by the percentage of ^{14}C-labelled hormone over the total counts recovered in the foetuses 15 min. after injection of 0.5 μCi ^{14}C-corticosterone into the mother.

Table VII shows the metabolism of corticosterone by the foetal tissues during the third trimester of pregnancy. In good agreement with the results in Table VI, a very high percentage of injected radioactive corticosterone was found in the inactive 11-dehydro form from days 12 to 14. Then, a dramatic change in the metabolism occurred around day 16, where most of the hormone was now recovered in the active form.

A transient change occurred on day 13 in the relative amount of corticosterone ($p=.02$) and 11-dehydrocorticosterone ($p<.01$), whereas day 14 was virtually identical to day 12.

TABLE VII. CORTICOSTERONE AND ITS 11-DEHYDRO METABOLITE IN FOETAL TISSUES

	% of Total Counts in Foetal Tissues ± SEM	
Gestational Day	cpd.B	cpd.A
12 (7)	15.5 ±1.6	84.6 ±1.5
13 (5)	24.5 ±3.9	73.5 ±3.1
14(7)	16.9 ±0.3	83.1 ±0.3
16 (8)	49.1 ±1.1	52.3 ±2.3
17 (8)	90.9 ±1.1	9.1 ±1.1

B. In vitro experiments

1. In vitro metabolism of corticosterone and 11-dehydrocorticosterone

The relationship between the dehydrogenation of corticosterone to 11-dehydrocorticosterone and the reduction of the latter to corticosterone can have a great effect upon the biological activity of corticosterone. In order to study the metabolic fate of the two compounds, minced placental and foetal tissues were incubated with ^{3}H-corticosterone and ^{14}C-11-dehydrocorticosterone. Gestational days 13, 14 and 15 were chosen because around these days, a great change in the conversion of corticosterone to 11-dehydrocorticosterone occurred, as indicated in Table VII.

The results in Table VIII show that 1) the placenta was the most active tissue for the reduction to corticosterone and this activity remained constant for the 3-day period; 2) the head was the most active in dehydrogenating corticosterone; and 3) the body showed reductase activity increasing with time of gestation.

TABLE VIII. IN VITRO METABOLISM OF CORTICOSTERONE AND 11-DEHYDROCORTICOSTERONE BY FOETAL TISSUES

	% of Dehydrogenation of Corticosterone ±SEM			% Reduction of 11-Dehydro-corticosterone ±SEM		
Gestational Day	Placenta	Head	Body	Placenta	Head	Body
13 (12)	20.7 ±1.0	62.6 ±1.5	61.1 ±1.9	83.4 ±2.5	8.8 ±1.4	8.1 ±1.2
14 (8)	16.2 ±1.0	61.0 ±1.3	49.0 ±4.7	88.0 ±1.6	7.3 ±0.6	12.0 ±1.4
15 (8)	18.5 ±1.8	46.5 ±1.2	27.9 ±1.1	86.9 ±2.0	14.0 ±0.8	37.5 ±1.9
$\frac{\text{reduction}}{\text{dehydrogenation}}$						
13	4.1	0.14	0.13			
14	5.4	0.12	0.24			
15	4.7	0.30	1.34			

2. Localization of the reductase activity

Since the weight of the foetal liver increases a great deal during the third trimester of pregnancy (20 mg at day 14; 70 mg at day 18) and since it is a target tissue for corticosterone (18), it was suspected that the reductase activity shown by the body was due to the liver. In order to test this, the foetal livers from gestational day 15 foetuses were removed from the carcasses and liver and carcass were incubated separately. It is evident, according to Table IX, that the liver effected a net reduction and the carcass exhibited a significant net dehydrogenation.

TABLE IX. REDUCTION OF 11-DEHYDROCORTICOSTERONE BY THE FOETAL LIVER AND RESIDUAL CARCASS

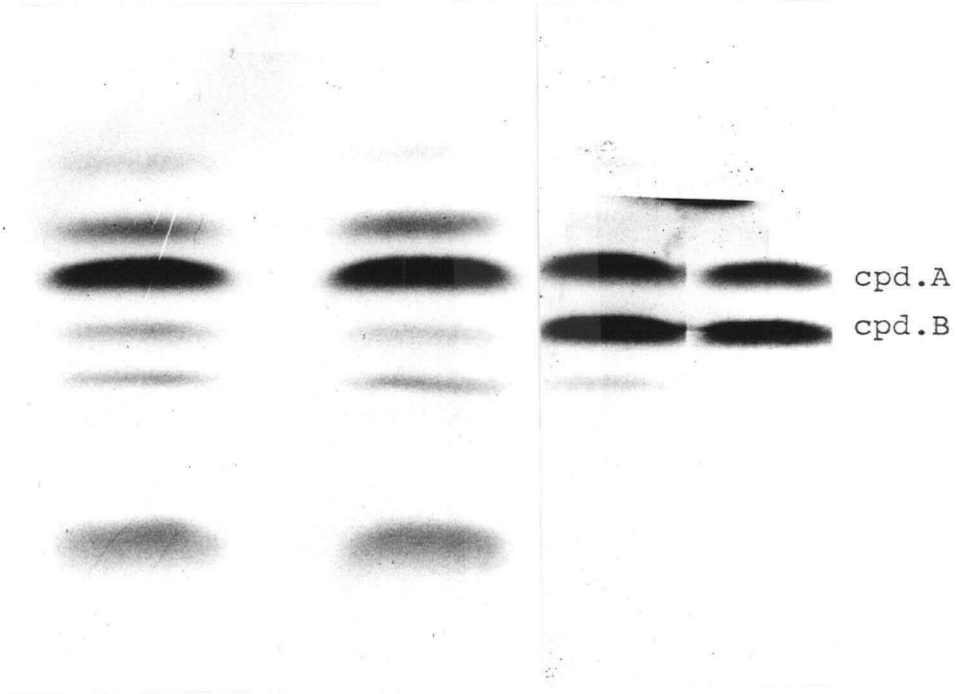
Reaction	% Conversion of 11-dehydrocorticosterone ±SEM	
	Liver	Carcass
Dehydrogenation (8)	21.6 ±1.3	29.9 ±2.7
Reduction (8)	48.4 ±1.4	12.5 ±1.3
Ratio $\frac{\text{reduction}}{\text{dehydr.}}$	2.24	0.42

3. Metabolism of corticosterone in the foetal head

During the in vivo experiments, autoradiograms (AR) were made of the TLC before elution to see if there were metabolites of corticosterone other than 11-dehydrocorticosterone in significant amounts. Whereas the AR of the TLC from the bodies did not show any detectable band, those from the heads showed many bands. They were faint, probably due to the low amount of ^{14}C accumulated in the head. In order to determine whether these bands could be reproduced, minced foetal heads were incubated with 10,000 cpm of chromatographically pure ^{14}C-corticosterone and the TLC were exposed to an X-ray film. Figure 2 shows that indeed corticosterone is metabolized extensively into several compounds by the foetal head. The in vitro metabolism was similar to that in vivo. The AR shows that between days 14 and 15, there is a dramatic change in the metabolism of corticosterone. At day 15 there is a decrease in the rate of metabolism of corticosterone to 11-dehydrocorticosterone.

Only the TLC zones from corticosterone and 11-dehydrocorticosterone have been eluted. Figure 2 footnote shows the percentage ^{14}C of the total for each zone. The remaining zones have not yet been identified.

FIGURE 2. METABOLISM OF CORTICOSTERONE BY THE FOETAL HEAD



The straight dark band at the top of the right-hand chromatogram is an artifact

Footnote:

Gestational Day	% of Total ^{14}C-dpm ±SEM	
	cpd.B	cpd.A
14 (8)	15.7 ±0.9	84.4 ±0.9
15 (4)	52.8 ±3.0	47.3 ±3.0

C. Enzyme kinetics

1. Cell fractionation and coenzyme specificity

In order to localize the subcellular fraction responsible for the reductase activity and to determine the coenzyme requirement, foetal livers were homogenized and the nuclear, mitochondrial and microsomal, as well as the crude homogenate and the final supernatant, were tested for reductase activity in the presence of NADH or NADPH. Whereas the enzyme activity seemed rather evenly distributed in the crude unwashed fractions (Table X), NADPH appeared to be the preferred coenzyme.

Since it was felt that washing the subcellular fractions would improve the localization of the enzyme activity, the fractions were washed in the same buffer and tested for activity. As Table XI indicates, the highest reduction was localized in the mitochondria. The preference for NADPH was very marked. The nuclear fraction showed a significant reductase activity, but it also had a substantial amount of broken cells that sedimented with the nuclei. Re-homogenization of the "nuclear" pellet yielded a more pure fraction, which activity decreased with washing.

TABLE X. ENZYME ACTIVITY AND COENZYME SPECIFICITY IN THE CRUDE SUBCELLULAR FRACTIONS

Subcellular Fraction	% Substrate Converted ±SEM	
	+NADH	+NADPH
Homogenate (3)	46.7 ±6.3	66.0 ±4.8
Nuclei (3)	41.3 ±0.8	53.5 ±3.7
Mitochondria (3)	56.5 ±3.4	63.3 ±4.6
Microsomes (3)	37.9 ±3.2	59.4 ±3.6
Supernatant (3)	26.9 ±2.1	86.3 ±3.2

Each incubation mixture contained the equivalent of 50 mg tissue

TABLE XI. ENZYME ACTIVITY AND COENZYME SPECIFICITY IN WASHED FRACTIONS AND THEIR WASHES

Subcellular Fractions	% Substrate Converted			
	Fractions		Washes	
	NADH	NADPH	NADH	NADPH
Nuclei	8.2	77.5	11.6	79.5
Re-homogenized nuclei	7.4	29.0	--	--
Mitochondria	8.6	95.2	12.6	85.1
Microsomes	5.0	11.1	7.3	11.2
Supernatant	6.9	30.3	--	--

Each figure is an average of determinations on two separate batches of enzyme

When mitochondria were kept in the buffer for more than 30 min., enzyme activity was released into the wash. In all cases, the coenzyme specificity for NADPH was very clear.

2. Development of the reductase activity in the foetal liver

Having localized the liver as a site of reduction, experiments were carried out to determine whether or not the presence of coenzyme was the limiting factor to the enzyme reaction. It was found that the addition of the coenzyme NADPH increased only slightly the reduction of 11-dehydrocorticosterone to corticosterone by minced foetal liver (Table XII).

TABLE XII. DEVELOPMENT OF THE REDUCTASE ACTIVITY IN THE FOETAL LIVER

Gestational Day	% Substrate Reduced ±SEM	
	Without NADPH	With NADPH
14	11.9 ±0.9 (5)	14.0 ±0.8 (6)
15	39.4 ±2.2 (4)	56.8 ±3.5 (5)

3. pH optimum

The pH optimum for reduction was determined by incubating a crude preparation at pH varying from 4.5 to 9. It was established to be between 5.5 and 6 (Figure 3). These two pH values gave the same activity.

4. Km

The reduction of 11-dehydrocorticosterone (Figure 4) showed a substrate inhibition occurring between 80 and 100 µM. The dehydrogenation of corticosterone (Figure 6) showed substrate inhibition at a lower concentration (between 15 and 20 µM).

The Km and Vmax for both reduction and dehydrogenation reactions were determined from the Lineweaver-Burk plot. For the reduction of 11-dehydrocorticosterone to corticosterone, the Km was 33 µM and Vmax, 0.4 µmoles/min./ml (Figure 5). For the reverse reaction, Km=10 µM and Vmax=0.21 µmoles/min./ml.

FIGURE 3. pH DEPENDENCE OF THE REDUCTASE ACTIVITY

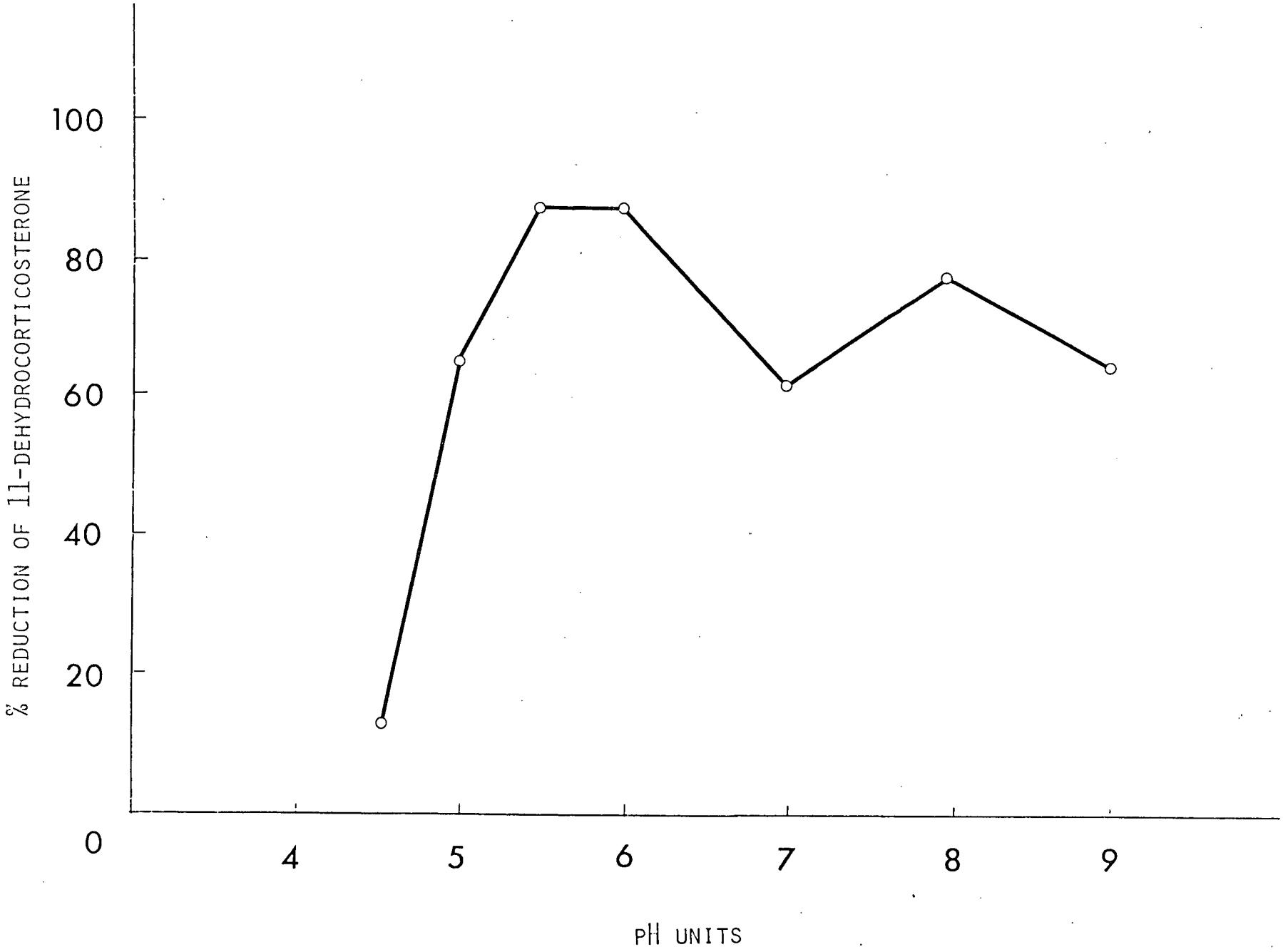


FIGURE 4. EFFECT OF CONCENTRATION OF 11-DEHYDROCORTICOSTERONE ON REDUCTASE ACTIVITY

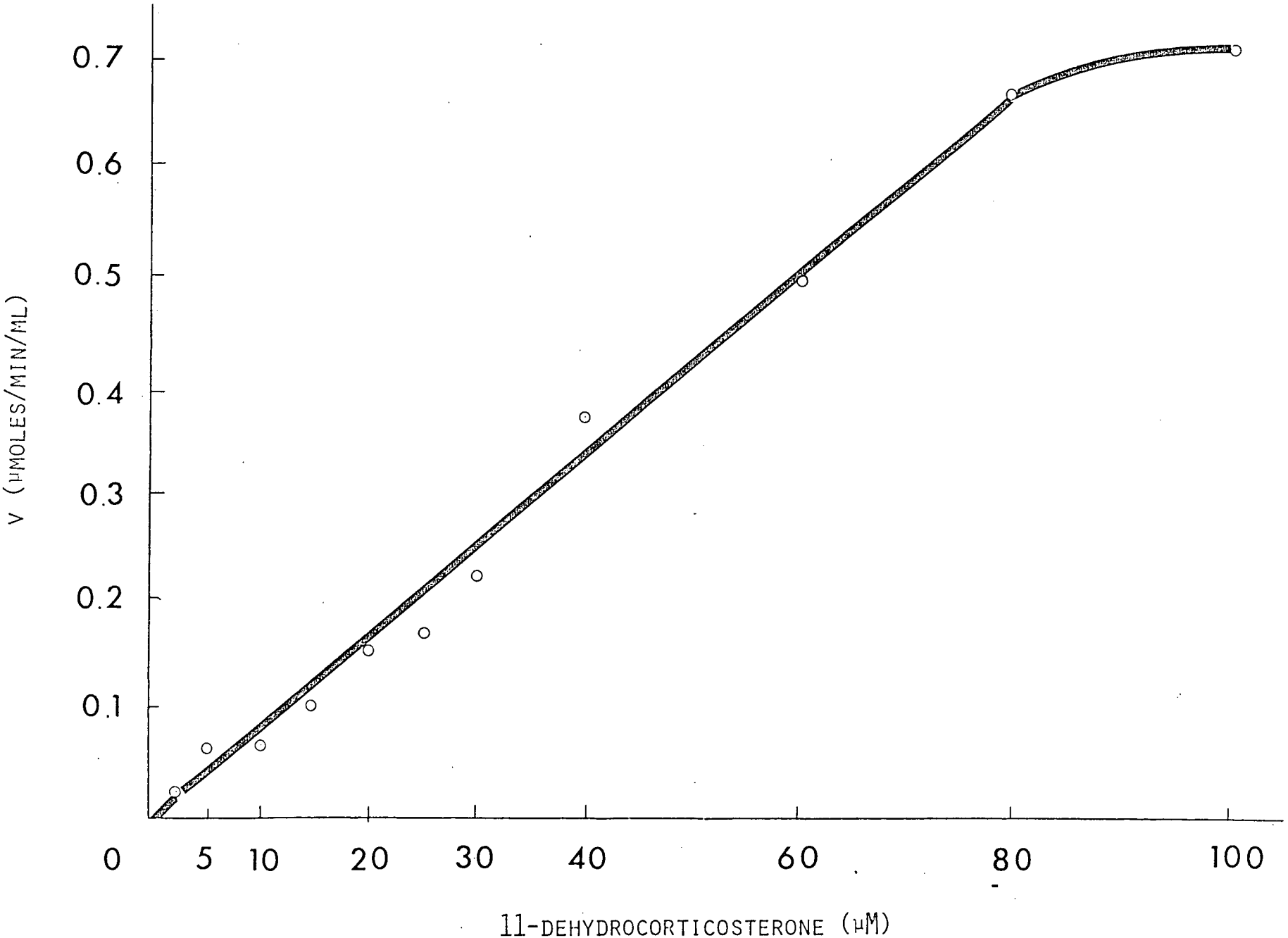
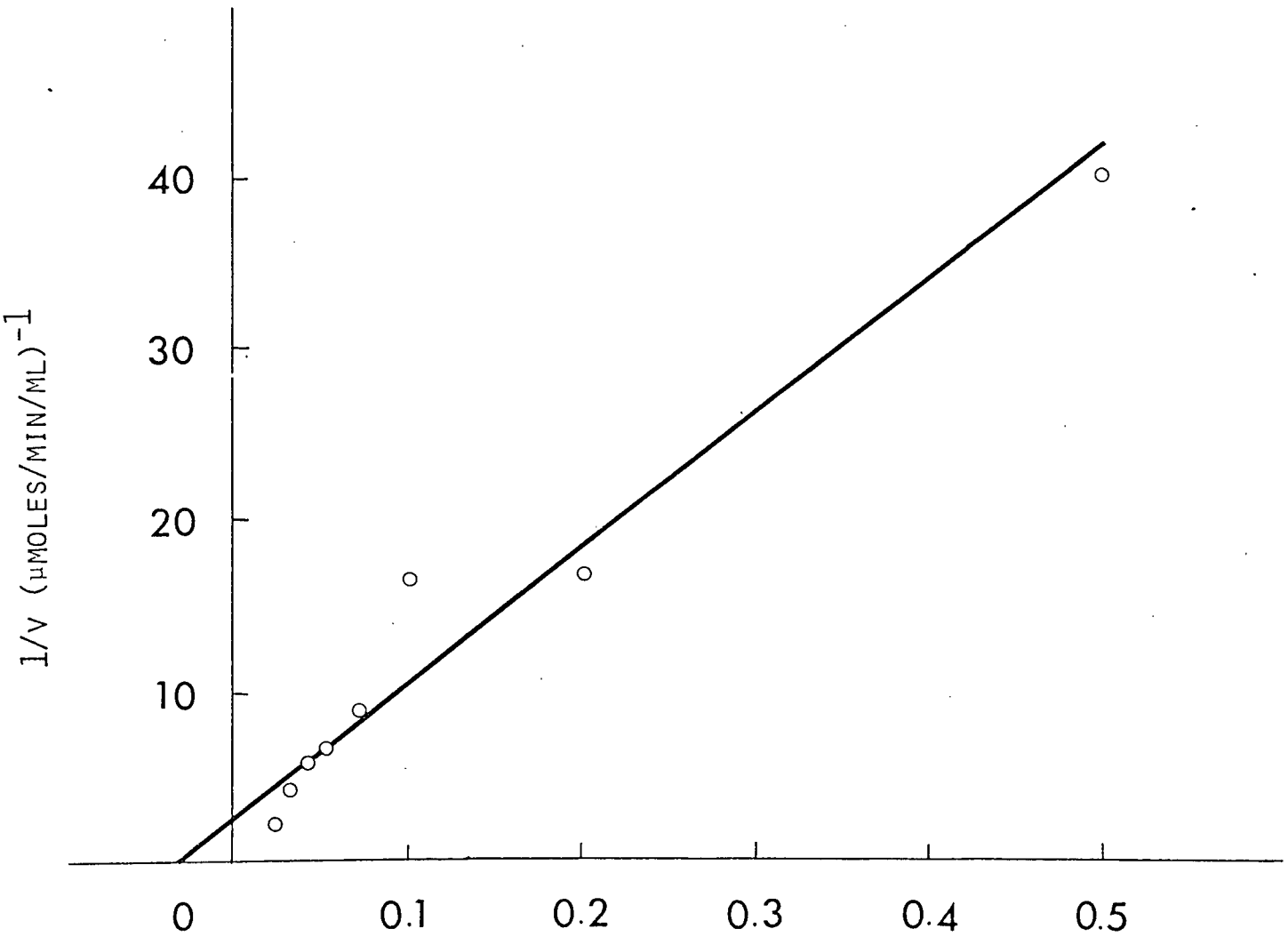


FIGURE 5. LINEWEAVER-BURK PLOT FOR REDUCTION OF 11-DEHYDROCORTICOSTERONE



K_M = 33 μM

V_{MAX} = 0.4 μMOLES/MIN/ML

FIGURE 6. EFFECT OF CONCENTRATION OF CORTICOSTERONE ON DEHYDROGENASE ACTIVITY

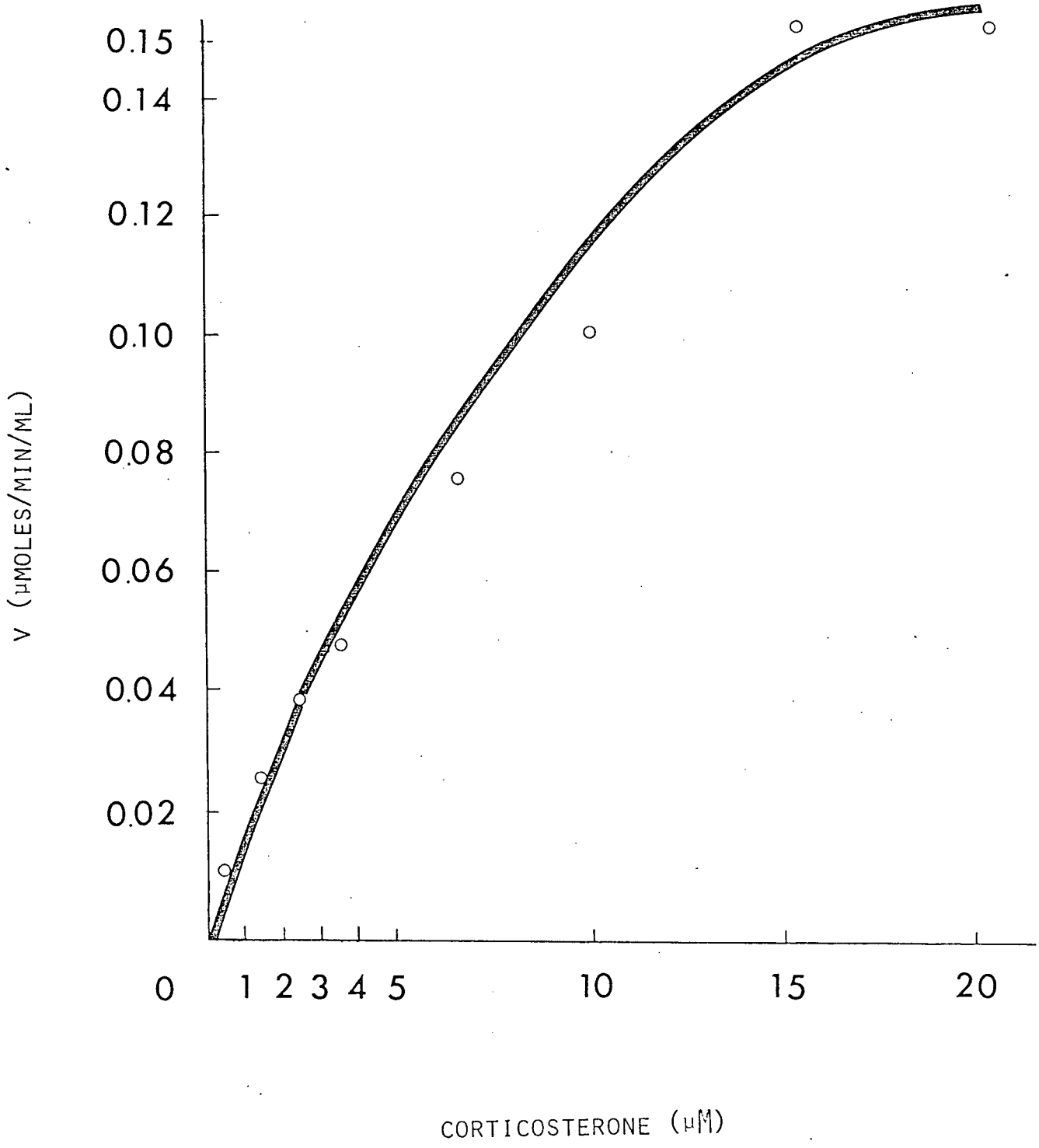
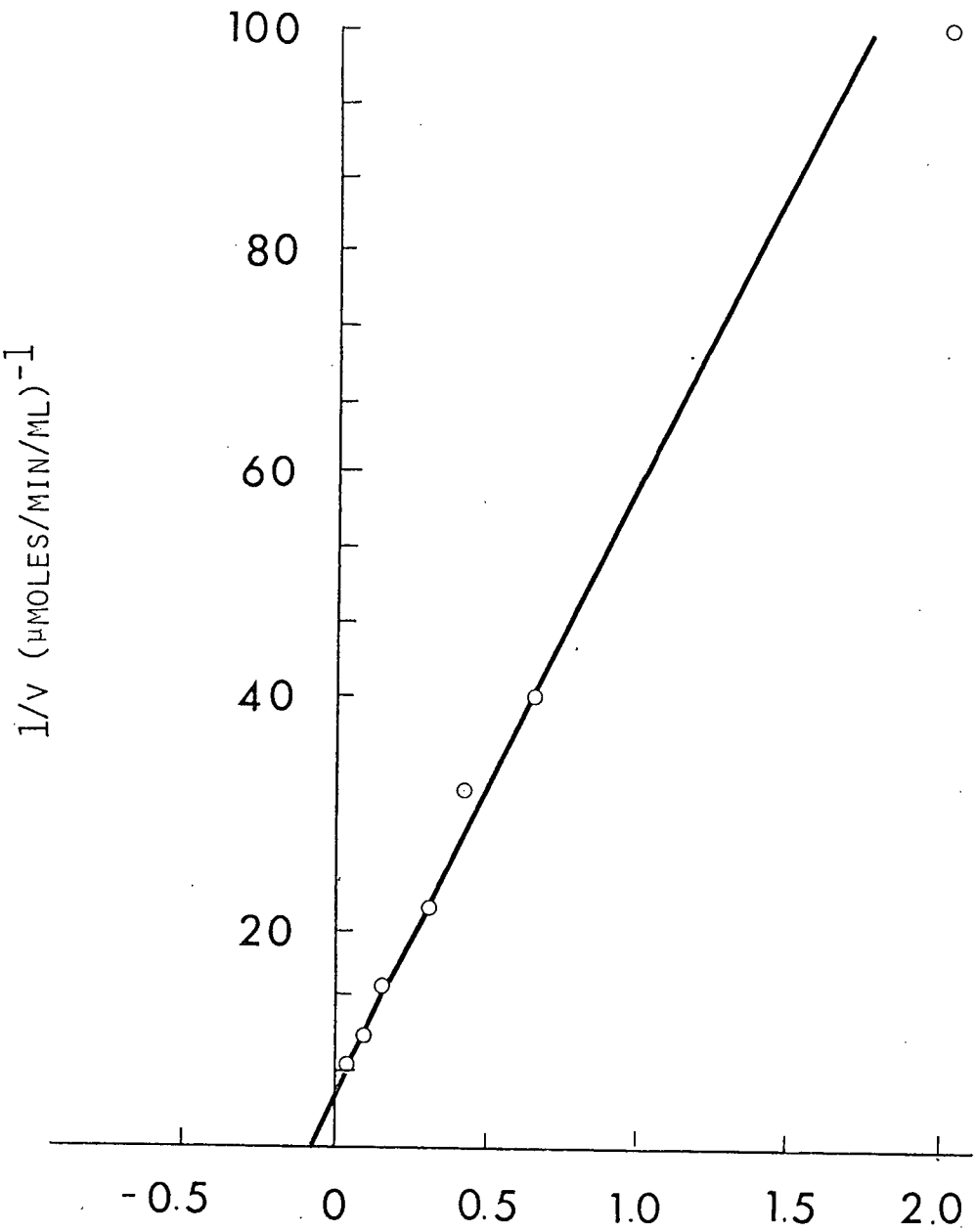


FIGURE 7. LINEWEAVER-BURK PLOT FOR DEHYDROGENATION OF CORTICOSTERONE



K_M = 10 μM

V_{MAX} = 0.21 μMOLES/MIN/ML

5. Content and specific activity of recovered ^{14}C-corticosterone in the foetal body

Since the metabolic activity of the head was greatly different of that of the body (Table VIII), the fate of ^{14}C-corticosterone was examined in the foetuses with the head removed during the critical period where the metabolism of corticosterone in the body changed drastically. As shown in Table XIII, there is a dramatic increase in specific activity at day 15, indicating an enrichment of the corticosterone foetal pool by the isotope. This is assumed to be due to the reduction of ^{14}C-11-dehydrocorticosterone.

TABLE XIII. CONTENT AND SPECIFIC ACTIVITY OF CORTICOSTERONE RECOVERED IN THE FOETAL BODY

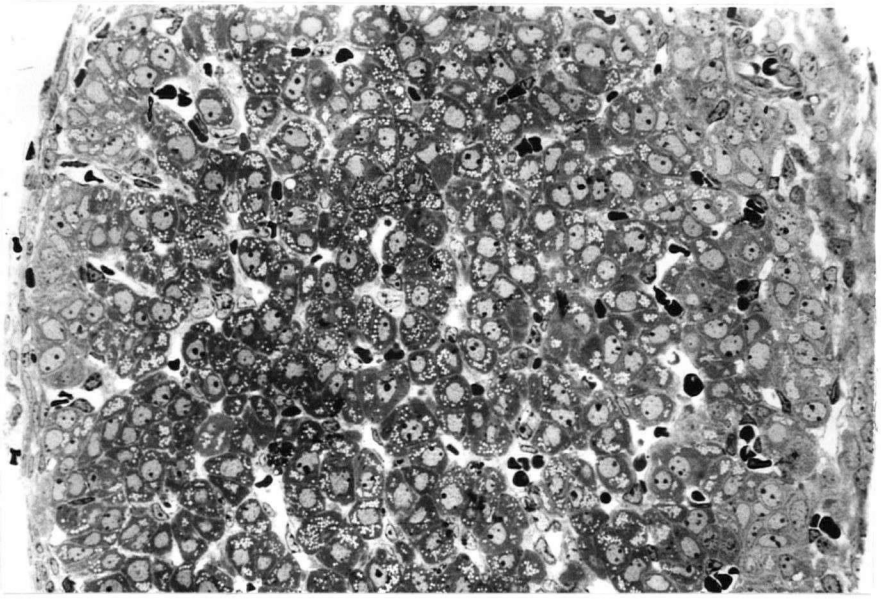
Gestational Day	ng cpd.B/g tissue ±SEM	^{14}C-dpm/ng cpd.B ±SEM
13 (5)	812.9 ±157.1	0.71 ±0.12
15 (6)	564.0 ±66.3	8.44 ±1.44

6. Histological studies

Corticosteroids are regulated in a complex manner in various foetal tissues. The effect of a synthetic steroid which, because of its structural modifications, escapes this metabolic regulation has been reported (40). Dexamethasone which is not bound by serum transcortin, accumulated more extensively in foetal tissue and underwent less metabolic alteration than corticosterone, the natural hormone in the mouse. Although high doses of corticosterone injected into pregnant mothers had little effect, dexamethasone was lethal to mouse foetuses. An attempt was made to find a basis for this toxicity. Foetal tissues and placenta were examined 4 and 20 hrs. after the 17-day-pregnant mother had been injected with 200 µg of dexamethasone. There was no evidence of histological changes.

Dexamethasone has been reported to cause involution of foetal adrenal cortex but to stimulate the development of the medulla and catecholamine synthesis (2). So foetal adrenals were examined under LM (Figure 8) and TEM (Figure 9). No significant difference was observed in treated adrenals, except possibly for a slight reduction in size of the gland itself.

FIGURE 8. LIGHT MICROGRAPHS OF FOETAL ADRENAL GLANDS OF NORMAL MICE (UPPER) AND THOSE FROM MOTHERS TREATED 20 HOURS PREVIOUSLY WITH 200 µg DEXAMETHASONE (LOWER) X 400



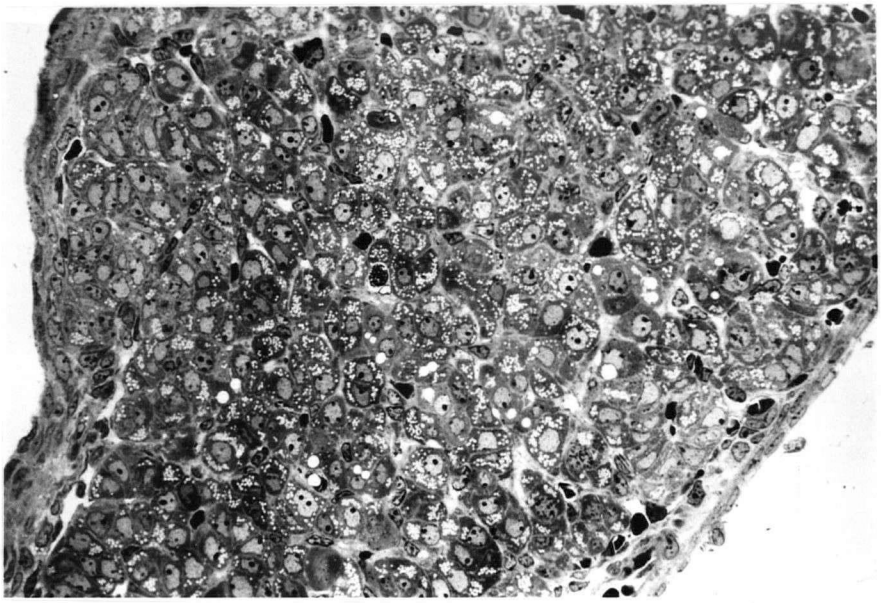
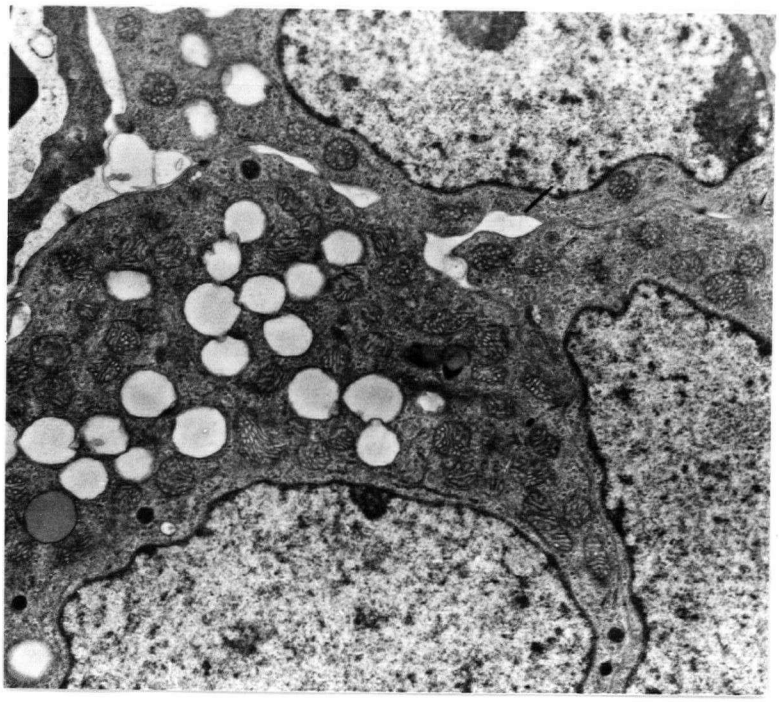
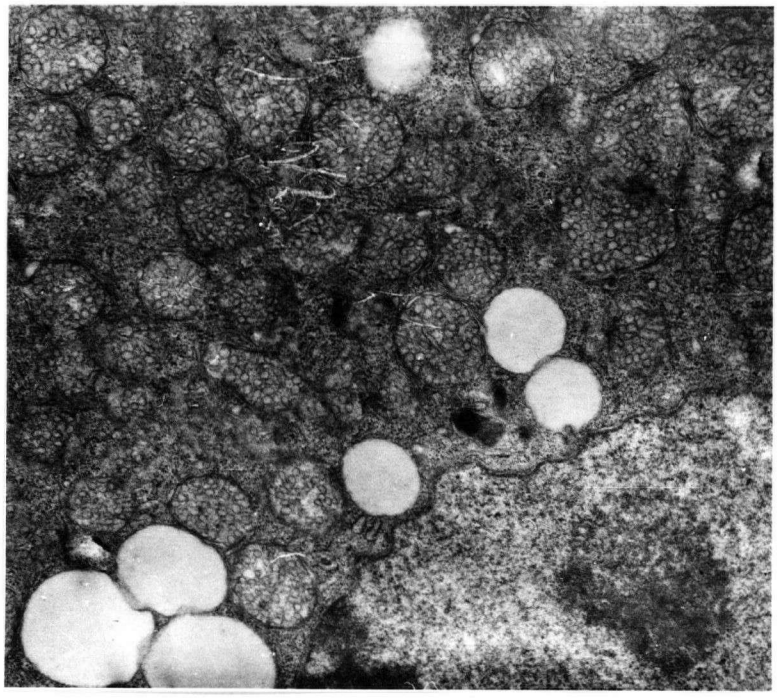


FIGURE 9. TRANSMISSION ELECTRON MICROGRAPHS OF FOETAL ADRENAL GLANDS OF NORMAL MICE (UPPER) AND THOSE FROM MOTHERS TREATED 20 HOURS PREVIOUSLY WITH 200 μg DEXAMETHASONE (LOWER)



X6000



X12000

DISCUSSION

The transport across the placenta of radioactive corticosterone revealed some interesting features of corticosterone metabolism in the foetus. From gestational days 13 to 17, the maternal corticosterone level increased, whereas on the foetal side, it decreased by half (Table II). This can probably be attributed to the increase which occurs at the time in the transcortin content of maternal blood (8). This protein binds corticosterone specifically and reduces its passage into tissues. On the other hand, the specific activity of injected corticosterone in the foetus actually increased somewhat (Table III). This suggests that there is no change in the foetal pool; e.g., by foetal adrenal production of corticosterone, between days 13 and 17. The content of foetal corticosterone on days 13 and 17 is similar to that reported on day 16 and determined by fluorometric analysis (35).

The role of the placenta as a barrier was dramatically shown by the large accumulation of counts recovered from the placenta and the foetal tissues (Table VI). From day 12 to 14, there was more than 75% of the labelled corticosteroids recovered in the two tissues, in the placenta. There was very little of the 11-dehydro metabolite of corticosterone in the placenta, whereas in the foetus, the hormone was extensively metabolized to 11-dehydrocorticosterone. From

these experiments (Tables VI and VIII), it appears that the placenta controlled the flow of hormone into the foetus, whereas the foetal tissues assumed different metabolic functions.

Whether or not the hormone found in the foetus is of foetal or maternal origin cannot be determined definitely from the present data (Table IV and V). Removal of maternal adrenals resulted in normal foetal levels of corticosterone. However, the specific activity of foetal corticosterone 15 minutes after injection was as high as one-quarter that in maternal blood, indicating rapid and extensive transfer from mother to foetus (Tables III and XIII).

During the third trimester of pregnancy, the metabolism of corticosterone on the foetal side changed greatly (Table VII). At the beginning of the trimester; i.e., from day 12 to 14, metabolism was extensive in the direction of formation of the 11-dehydrometabolite of corticosterone, which represented around 80% of all counts recovered in the foetal tissues. Then after day 14, there was a drastic change in the dehydrogenation of corticosterone and by day 17, over 90% of the hormone was recovered in the active form. Hence, there is evidence that the foetus can metabolize corticosterone actively following a definite pattern during the last trimester of pregnancy.

Not only can the foetus metabolize corticosterone actively, but this metabolism is compartmentalized (Table VIII). The in vitro metabolism by the placenta, the foetal bodies or the foetal heads from gestational days 13, 14 and 15 showed that the placenta played a constant and very active role in reducing 11-dehydrocorticosterone. From these days, the metabolism by the head was definitely towards the inactivation of corticosterone, whereas the body exhibited a very strong dehydrogenase activity at day 13; this decreased by one-half by day 15. It appears then that within the foetus itself, corticosteroids are metabolized quite differently in different tissues.

The reduction of 11-dehydrocorticosterone became the centre of interest of this work, so efforts were made to localize and study the development of the reductase activity. It is evident, according to Table IX, that the foetal liver was the major site of reduction. The residual activity effected by the carcass might be attributed mostly to the foetal lungs which are another target tissue for corticosterone and since reductase activity has been observed in the human lung (36). In the same system, the dehydrogenation of corticosterone was higher in the residual carcass, probably due to the foetal kidneys.

When foetal liver on days 14 and 15 was incubated with 11-dehydrocorticosterone, addition of the coenzyme NADPH

increased only slightly the conversion to corticosterone (Table XII), indicating that the coenzyme was not likely the limiting factor to the reaction, but rather an increase in enzyme concentration was responsible for the conversion to the active hormone.

Since it has been shown that there was a definite change in corticosteroid metabolism in the foetus during the third trimester of pregnancy (Table VII) and that the liver was the major site of reduction, it was difficult to explain that the specific activity of injected corticosterone changed only slightly from day 13 to 17 in whole foetal tissues. An experiment was designed to study the fate of injected ^{14}C-corticosterone in the headless body, where the reduction is prominent. Table XIII shows that there was an 11-fold increase in specific activity at day 15. This could not be due to an increase in transport of the hormone from the maternal side, since the specific activity would have remained constant. It could not be attributed to a change in foetal pool size of corticosterone either, since the amount of hormone in the body was in the same order of magnitude as that of the whole foetus. It is postulated that this sharp rise in specific activity was due to the enzymatic reduction by the foetal liver of labelled 11-dehydrocorticosterone which was itself the product of the dehydrogenation of the injected labelled corticosterone; i.e., injected ^{14}C-corticosterone $\xrightarrow{\text{dehydrogenation}}$ ^{14}C-11-dehydrocorticosterone

reduction → ^{14}C-corticosterone, enriching considerably the foetal pool of labelled corticosterone.

The results from the incubation in vitro of the foetal brain (Figure 2) give additional evidence of the compartmentalization of corticosteroid metabolism in the foetus. Many unidentified metabolites have been detected by AR of TLC from the head, which were not present on those from the body. No attempt has yet been made to identify these zones, but here too there is evidence of a striking change between days 14 and 15, with a decrease in the formation of the unknown metabolites as well as in the dehydrogenation of corticosterone.

The specificity for NADPH as coenzyme agrees well with reports in the literature (37, 38) for a similar enzyme in other tissues. The pH optimum was found to be on the acid side; i.e., between 5.5 and 6. A dip in the reductase activity was observed at pH 7 ($p<.02$), the same pH at which Wong (39) also observed a dip for the dehydrogenation of corticosterone by adult mouse liver and placenta.

This work has pointed out the importance of the 11β-hydroxysteroid:NADP oxidoreductase in the foetal liver during the third trimester of pregnancy in the mouse. The first outstanding feature is the compartmentalization of the oxidoreductase activity. Not only was the foetus capable of

metabolizing corticosteroids, but there was a definite pattern of metabolism in different organs. The placenta metabolized the steroid hormone and also appeared to assume a regulatory control over the flow of steroid from the maternal to the foetal side.

In the human, the major site of dehydrogenation of corticosteroids is the placenta, where maternal cortisol is largely converted to cortisone, the predominant corticosteroid in foetal and cord blood (11, 16, 29). In the mouse the head appears to be the most active site (Table VIII). In both, the liver is a site of reduction of the metabolite to the 11β-OH form of the active hormone (16, 18 and Table IX). Burton has found this reductase activity in human foetal liver as early as 20 weeks (16). In foetal mouse liver the appearance of glycogen deposition coincided with the appearance of reductase activity (18). In the human lung, another "target" organ of corticosteroids, reductase activity has also been demonstrated (36).

An alternate explanation can be offered for the observations of Murphy (10, 11), who found consistently increased cortisol levels in human foetal arterial blood as compared with umbilical venous blood entering the foetus. She attributed this to production by the foetal adrenal. However, this could as easily be due to reductase activity in foetal liver and lung, which reduces a portion of the abundant

cortisone which is present. Thus, the changes in the amount of active hormone could be determined not by the foetal glands but rather by changes in the metabolism of maternal steroids, with each tissue showing its own distinct pattern of metabolism.

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