ADRENAL CORTICOSTEROID BIOGENESIS AND METABOLISM IN THE HUMAN NEWBORN INFANT

Donald Arthur Hillman

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Endocrine Research Laboratory
Montreal Children's Hospital,
Department of Investigative Medicine
McGill University, Montreal

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LIST OF ABBREVIATIONS

ACTH:	adrenocorticotrophic hormone
Aldosterone:	11β, 21-dihydroxy-18-al-pregn-4-ene-3,-20-dione
Androstenedione	androst-4-ene-3, 17-dione
C-19 steroids	steroids with 19 carbon atoms
C-20 steroids	steroids with 21 carbon atoms
Cholesterol	cholest-5-en-3β-ol
Corticosterone	11β, 21-dihydroxypregn-4-ene-3, 20-dione
Cortisol	11β, 17α, 21-trihydroxypregn-4-ene-3, 20-dione
Cortisone	17α, 21-dihydroxypregn-4-ene-3, 11, 20-trione
c.p.m.	counts per minute
Dehydroepiandrosterone	3β-hydroxyandrost-5-en-17-one
11-dehydrocorticosterone	21-hydroxypregn-4-ene-3, 11, 20-trione
Δ^4 or Δ^5	double bond between C-4 and C-5 or C-5 and C-6
11-desoxycortisol	17α, 21-dihydroxypregn-4-ene-3, 20-dione
Desoxycorticosterone	21-hydroxypregn-4-ene-3, 20-dione
DPN	diphosphopyridine nucleotide
d.p.m.	disintegrations per minute
Estradiol	estra-1, 3, 5(10)-triene-3, 17β-diol
Estriol	estra-1, 3, 5(10)-triene-3, 16 , 17β-triol
Estrone	3-hydroxyestra-1, 3, 5(10)-trien-17-one
Etiocholanelone	3 -hydroxy-5β-androstan-17-one
11β-hydroxyandrostenedione	11β-hydroxyandrost-4-ene-3, 17-dione
17 -hydroxyprogesterone	17α-hydroxypregn-4-ene-3, 20-dione
17-hydroxypregnenolone	3β, 17α, -dihydroxypregn-5-en-20-one

6β-hydroxycortisol	6β, 11β, 17α, 21-tetrahydroxypregn-4-ene-3, 20-dione
µg	microgram
µmole	micromole
Pregnanediol	5β-pregnan-3α, 20α-diol
Progesterone	pregn-4-ene-3, 20-dione
Tetrahydrocortisol	3α, 11β, 17α, 21-tetrahydroxy-5β-pregnan-20-one
Tetrahydrocortisone	3α, 17α, 21-trihydroxy-5β-pregnane-11, 20-dione
TPN	triphosphopyridine nucleotide
Tris	tris(hydroxymethyl) aminomethane

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Structural changes in the human newborn adrenal and some evidence of atypical responsiveness to stress during the first week of life have stimulated much experimentation and speculation. It is probable, although not positively proven, that these changes reflect both withdrawal of the newborn from metabolic factors taking place within the foetoplacental unit and adaptation to extra-uterine life.

REVIEW OF THE LITERATURE

1. ADRENOCORTICAL DEVELOPMENT AND FUNCTION IN THE HUMAN FOETUS.

A. EMBRYOLOGY

The anatomical characteristics of the foetal adrenal are: first, its large size in relation to total body weight, and second, the presence of two distinct cellular "zones". During most of pregnancy the foetal zone comprises about 80% of the total adrenal volume, while the adult zone consists of a very thin layer of small cells lying directly beneath the capsule. The difference in the histological appearance of the two zones has led to speculation about their embryological origin.

Keane and Hewer (2), and more recently Uotila (3) and Lanman (4), have proposed that the embryological origin and development of the foetal zone is different from that of the adult zone. In the 10 mm embryo Keane and Hewer observed the migration of cells from the ceolomic epithelium into the mesenchyme. These cells, which at this stage of development were surrounded by a capsule derived from the mesonephros, were believed to give rise to the foetal zone. At a later stage a second migration of cells from the ceolomic epithelium was observed. These cells, which were invading the capsule and encircling the foetal cortex, would eventually develop into the adult zone. These authors also emphasized that differences in the staining characteristics and growth patterns of the foetal and adult cortex support the concept that these zones are embryologically different.

As opposed to this view, Crowder (5), from an examination of the Carnegie embryological collection, concluded that the cells of the foetal and adult cortex are both derived from the ceolomic epithelium and that the development of the foetal adrenal occurs as the result of a single cell migration. A continuous production of new cells was observed in the outer zone. These cells subsequently migrated and matured in the deeper zones. Thus differences in the maturity of the cells accounted for the differentiation between the adult and foetal zones. This theory has been supported by Gruenwald (6) who described the gradual transition from the small subcapsular granulosa cells into the larger cells of the foetal zone. The electron microscopic studies of Ross (7) also suggest that the adult zone of the foetal adrenal may be a germinative zone. He noted that the cells of the adult cortex were undifferentiated and contained an abundance of free granules of ribonucleoprotein. These free granules which are in general associated with rapidly dividing cells, are related to synthesis of cytoplasmic protein.

There remain, then, two theories of the embryology of the foetal adrenal: the foetal zone and the adult zone may be derived from a different cell migration, or the two zones may arise simultaneously from a common cell of origin. The weight of histological evidence tends to favour the latter theory.

B. PATTERN OF GROWTH

The growth pattern of human foetal adrenals has been described in several studies (8-14). By the fourth month of gestation the adrenals reach their maximal size relative to total foetal

weight and at that time are as large as the kidneys. Although during the second half of pregnancy their relative size decreases gradually, the adrenals of the newborn are proportionately twenty times larger than adult adrenals. From a study of 91 foetuses, Ekholm (10) has reported that the adrenal weight, expressed as a percent of total body weight, is 0.5% at three months of foetal development, and 0.25% at birth, whereas it is only 0.01% in the normal adult.

From the second month of gestation the adult zone consists of tightly packed small cells which resemble those of the zona glomerulosa and are arranged in arcades (2,7). As they extend centrally these arcades form short columns similar to those observed in the zona fasciculata. Beneath the adult zone lies the homogeneous foetal zone which is organized in a loose, reticular pattern. Its cells are larger than those of the zona reticularis which occupies the corresponding position in the adult gland.

Several reports (2,5,9) describe degenerative changes in the foetal zone as early as ten weeks prior to birth, while others (16,14,4) indicate that extensive foetal zone degeneration does not occur prior to birth, whether in full term or in premature infants.

The anatomical zonation of the foetal adrenal and the involutional changes of the foetal zone during the newborn period were first described in 1911 by Kern (17) and by Elliott and Armour (18). Tahka's (15) study of adrenal histology during the first two years of life indicates that the width of the foetal zone decreases rectilinearly up to the age of 21 days. Thereafter the area occupied by the foetal zone is replaced by a broad band of connective tissue

which gradually contracts, and after the 4th month of life persists only as a capsule surrounding the medulla. The permanent cortex develops rapidly after birth, doubling its width during the first three weeks of life.

Although delay in the involution of the foetal zone has been described in infants with a variety of diseases (14,15,16) studies by Lanman (4) and Tahka (15) have failed to substantiate this.

Early investigations of the adrenogenital syndrome led to the belief that the hyperplastic zona reticularis was due to the persistence of the foetal zone. Blackman (19) however has suggested that while the foetal zone cells may develop and differentiate into the cells of the post-natal zona reticularis, these zones are not identical. The cells of the foetal zone are much larger and the sinusoidal capillaries are wider than in the zona reticularis. This author also noted that in infants dying with congenital adrenal hyperplasia the involution of the foetal zone coexists with the hyperplasia of the zona reticularis.

A marked reduction of adrenal size and the virtual disappearance of the foetal zone are known to occur in anencephalic monsters. As a rule, in this congenital malformation of the central nervous system the pituitary gland is absent or hypoplastic (20-24). It is of interest to note that up to the fifth month of development the adrenal cortex of these foetuses appears normal in size and structure, suggesting that early foetal adrenal growth is independent of the pituitary.

In summary, the growth pattern of the foetal zone is characterized by two main features: rapid and extensive development up to the time of birth and involution immediately after birth. The gradual growth of

the adult zone postnatally accounts for the entire structure of the adrenal cortex in the adult.

C. HORMONAL CONTROL

The factors responsible for the control of adrenal growth and function are not clearly established. Jones (25) has postulated that chorionic gonadotrophin was the foetal adrenotrophic factor. However Lanman (26) finds this thesis untenable on the grounds that in spite of the high levels of chorionic gonadotrophin in the maternal circulation this hormone is not detected in foetal blood or urine.

Lanman has conducted a series of experiments in which he has administered ACTH, estrogens, or chorionic gonadotrophin postnatally to infants with fatal malformations. Only in two infants who had received ACTH was it possible to preserve the foetal zone.

On the basis of these studies Lanman concludes that the adrenotrophic properties of chorionic gonadotrophin, growth hormone, androgens, and estrogens are insufficient to explain the degree of adrenal enlargement found in the human foetus. He suggests that ACTH from the foetal pituitary controls the growth of the foetal zone. The most convincing evidence in favour of this thesis is the marked adrenal atrophy seen in the newborn infant with anencephaly (20-24).

In further support of this thesis Lanman cites the experiments of Symington (27) in which the adrenals were removed from adults after a four day period of treatment with ACTH. These adrenals were grossly enlarged and the cells at the level of the medullary border of the cortex were increased in size and had a homogeneous eosinophilic appearance very similar to the cells of the developing foetal zone.

These studies leave unexplained the functional significance of the growth pattern and the cause of the involution and degeneration of the foetal zone occurring immediately after birth.

Studies of comparative anatomy have helped but little to resolve these questions, for although adrenal structural changes have been reported in a number of laboratory animals during the newborn period these changes are not strictly comparable to those observed in the human. Recently however Benirschke (31) has reported that during foetal and neonatal life the adrenal of the marmoset monkey undergoes changes very similar to those of the human adrenal. It is possible that further studies on this primate may in the future lead to a better understanding of the human foetal adrenal.

D. ENDOCRINE FUNCTION

One source of uncertainty about the factors affecting the growth and involution of the foetal adrenal is our lack of knowledge regarding the function of the gland.

1. Morphological evidence of steroidogenesis.

Although the gross distribution of lipids in the adrenal cortex may be related to steroid formation (32,33), studies of the size and distribution of lipid inclusions have not been rewarding in the assessments of the functional activity of the newborn adrenal cortical cells (15). More informative data can be obtained if one considers the arrangement of adrenal cells. Careful light microscopy reveals that the cells of the adult zone of the foetal adrenal are very tightly packed, with little intracellular space and poor capillary distribution. In contrast the cells of the foetal zone are large and the cords of cells are separated by wide sinusoidal capillaries (7). Under the electron microscope the cells of the adult zone have scant cytoplasm,

a prominent nucleus, and their mitochondrial structure is not that of functionally active adrenal cells. The cells of the foetal zone contain a very large and prominent endoplasmic reticulum, a structure which is consistent with active lipid metabolism (34).

Based on these morphological characteristics Ross (7) has concluded that the foetal zone appears to be active in the production and secretion of steroids, while the adult zone serves as the germinative area of the foetal adrenal cortex.

2. Extractable steroids.

Very little preformed steroid is present in the adrenal. Rogers and Williams (35) and Bongiovanni (36) have found the corticosteroid content of normal adult adrenals to be between 5 and 10 μg/gm of tissue. In extracts of adrenals obtained from human foetuses between 9 and 21 weeks of gestation Bloch (37) tentatively identified three C-19 steroids as androstenedione, dehydroepiandrosterone, and 11β-hydroxyandrostenedione. The concentration of these steroids decreased from 30 μg/gm of adrenal at the 21st week. Trace amounts of cortisol were detected only in the glands obtained from foetuses over 16 weeks of gestation. An extract possessing sodium-retaining activity by bioassay was found in all the foetal adrenals tested. From these studies the authors postulated that during the first half of pregnancy the foetal cortex was primarily the site of C-19 androgen synthesis, and that the C-21 glucocorticoids were produced in small amounts by the adult cortex. Gardner and Tyce (38) extended Bloch's study by extracting the adrenals obtained from 12 newborn infants, and identified cortisol but could not find detectable amounts of progesterone or corticosterone.

The significance of these studies is difficult to interpret in terms of steroid secretion.

3. In vitro steroidogenic capacity.

Incubation of adrenal slices and whole homogenates provide a more direct means of investigating the potential function and biosynthetic capacity of both normal and abnormal adrenal tissues. Indeed several studies (39,40,37) have shown that following the incubation of normal adrenal, adrenal adenoma and hyperplastic adrenal the relative amounts of cortisol, corticosterone and 11-desoxycortisol produced into the incubation media were roughly comparable to the amounts of these steroids present in the adrenal vein blood.

In vitro studies of the human foetal adrenal have not consistently demonstrated steroid production unless precursors were added to the incubation media. Therefore such data reflect the presence of enzyme activities rather than providing evidence of internal steroid secretion by the gland.

The formation of cholesterol from acetate was demonstrated in adrenal slices from newborn infants by Davis (41). This observation was confirmed by Bloch (42) in isolated foetal zone obtained from foetuses of 12 to 23 weeks gestation.

The most concrete evidence that steroid biogenesis in the foetal adrenal may differ from that in the adult adrenal are the in vitro observations of Lanman (43), Villee (44,45) and Bloch (42) who showed that up to the 20th week of gestation the foetal adrenals possess a limited capacity to convert 3β-hydroxy-Δ^5 steroids into the corresponding Δ^4 3-ketones. These studies indicate a significant reduction of 3β-hydroxysteroid dehydrogenase activity, and are indirectly supported by the work of Mancuso (46) who demonstrated that the perfused previable foetus aromatizes testosterone and androstenedione but is unable to metabolize dehydroepiandrosterone.

The sequential hydroxylation of progesterone leading to the formation of cortisol has been studied in foetal adrenal preparations (44). 17 - hydroxylation is apparently well developed in very early embryonic life. In 4 cm. foetuses Villee demonstrated the conversion of progesterone to 17-hydroxyprogesterone and during the second quarter of gestation Solomon (47) demonstrated this conversion in isolated foetal zone.

Based on a series of incubations utilizing the adrenals of foetuses of different gestational ages Villee (44) observed that 21-hydroxylase was absent or inactive prior to the age of 10 weeks, and that 11-hydroxylase activity could not be positively demonstrated until the 13th week of gestation. Villee has also demonstrated the presence of 16-hydroxylase activity in all of the foetal adrenal tissues studied.

Further support of the results obtained by in vitro incubation of foetal adrenals is provided by Bird's (48) study of steroid metabolism in the perfused previable human foetus. 11,16,17 and 21-hydroxylation of perfused progesterone occurred in intact but not in adrenalectomized foetuses suggesting that these hydroxylases were present in the adrenal.

4. Foetal contribution to estrogen production.

Recently there has been much speculation as to the contribution of the foetus to the synthesis and metabolism of estrogens during pregnancy. As early as 1951 Lelong (49) proposed that part of the estrogens excreted in pregnancy urine was elaborated by the foetal adrenal. However Diczfalusy's initial studies rejected this role of the foetal adrenal, suggesting that the foetus was active only in estrogen metabolism and that foetal tissues including lung, intestine and liver had a great capacity to conjugate estrogen as sulfate esters (50). In subsequent studies Diczfalusy (1) and Bolté (51) showed that the principal pathway of estrogen synthesis in human pregnancy involves placental aromatization

of C-19 androgens originating from the foetus, and probably from the foetal adrenals. This concept offers a satisfactory explanation for the greatly diminished estrogen excretion in women pregnant with anencephalic monsters (52,53). It is also supported by Frandsen's (54) observation that low maternal estriol excretion during the last fifteen weeks of pregnancy is associated with very high foetal mortality and that a maternal estriol excretion of less than 3 mg per day is indicative of foetal death.

SUMMARY

It has not been definitely established whether the foetal zone and the adult zone have the same embryological origin but recent studies support the concept that the origin of both parts of the gland is identical.

Hormonal control of the foetal adrenal remains a matter of speculation. There is evidence suggesting that ACTH from the foetal pituitary is responsible for its development but the cause of its involution following birth remains obscure.

Histological studies, extraction and in vitro incubation of foetal adrenals show that this tissue is capable of steroid biogenesis. It is probable that the pattern of steroidogenesis is related to a gradual maturation of enzymatic activity that occurs with foetal development. Several reports describe a marked impairment of 3β-hydroxysteroid dehydrogenase activity in the adrenals of foetuses during the first half of gestation. This is believed to orient steroidogenesis towards the production of "primitive" 3β-hydroxy-Δ^5 androgens which are used by the placenta as one of the main sources of estrogen precursors.

II. MATERNAL FACTORS

The weight of the maternal adrenal is not increased during pregnancy and there is no histological or histochemical evidence to suggest that the gland is hyperactive (55). Studies of pregnant women with adrenocortical insufficiency demonstrate that the foetus and placenta do not contribute significant amounts of adrenocorticoids to the mother (56). Yet, in spite of these facts, changes occur in certain parameters of adrenocortical function during pregnancy which were originally thought to be consistent with a state of adrenocortical hyperactivity (57,58).

A. URINARY STEROID EXCRETION

Although urinary glucocorticoids are significantly increased in the course of a normal human pregnancy (58) the excretion of 17-hydroxycorticosteroids measured by the Porter-Silber reaction is comparable to that observed in non-pregnant women (59).

Appleby (60) and Steinbeck (61) found that while the excretion of the main metabolites of cortisol are essentially unchanged, that of the 17-hydroxy-21-desoxycorticosteroids measured by the method of Norymberski (60) are greatly increased. Steinbeck (61) suggests that this is mainly due to metabolites of 17-hydroxyprogesterone which may itself be a placental conversion product of progesterone. An increase in the urinary excretion of free cortisol and cortisone and a decreased conjugation of their respective tetrahydro derivatives with glucuronic acid have been reported by Venning (62) and Migeon (63).

Fantz (64) has described a progressive rise in the urinary excretion of 6β-hydroxycortisol in the course of pregnancy which he suggests to be a product of the peripheral metabolism of cortisol (65).

Secretion of 6β-hydroxycortisol by the adrenal cortex, as demonstrated in vitro by Touchstone (66), may also be possible.

Quantitatively there is no remarkable change in 17-ketosteroid excretion throughout pregnancy (67,68). Qualitatively, however, the proportion of 11-oxygenated 17-ketosteroids is increased while that of dehydroepiandrosterone, etiocholanolone and androsterone is decreased (69-72).

The secretion rate (75) of aldosterone and in consequence the excretion of this hormone and its metabolites (73,74) are increased but its metabolism remains unaltered (75). On the grounds that progesterone inhibits the sodium-retaining action of mineralocorticoids it is postulated that the increased aldosterone secretion of pregnancy serves to offset the sodium diuretic effect of the large quantities of progesterone and progesterone metabolites present in the maternal circulation (76,81)

Since Brown's demonstration of an increased estrogen excretion in pregnancy urine (77) it is generally believed that the main source of these steroids is the placenta (78). From a critical review of the literature and from his own studies (reported in other sections of this review) Frandsen (23) has concluded that estrogen production in pregnancy is due to the combined action of the foetal adrenal and the placenta and is not related to maternal adrenal function.

B. PLASMA ADRENAL STEROIDS

1. 17-hydroxycorticosteroids.

During pregnancy, in contrast to the relatively minor changes in 17-hydroxycorticosteroid excretion there occurs a progressive increase in plasma concentration of these steroids to values which at the time of delivery are three to four times greater than the average normal adult

value (57,67,79,80). Bro-Rasmussen (82) has demonstrated that this is due chiefly to an increase in plasma cortisol. Migeon (83) found that during the last trimester of pregnancy the half-life of ^{14}C cortisol is twice as long (160 minutes) as that observed in non-pregnant women. The above reports would suggest a state of hypercorticism, yet the cortisol secretion rate is normal (83). The explanation of these apparently conflicting results is provided by the demonstration of a rise of the plasma concentration of cortisol binding protein (transcortin) (84-88). During the third trimester of pregnancy there is a slight but significant increase in the concentration of non-protein bound cortisol. Since only the non-protein bound cortisol appears to be biologically active it was suggested (84) that cortisol binding to transcortin serves to prevent the development of hypercorticism.

Cohen (89) has described the alteration in the distribution of exogenously administered cortisol during pregnancy , resulting in a greater retention of the steroid within the vascular compartment.

It appears therefore that increased transcortin binds a greater amount of cortisol in the circulation,thus elevating the plasma cortisol concentration,and by retaining this steroid in the intravascular compartment impedes its metabolic degradation.

Increased transcortin concentration is apparently due to the high level of circulating estrogens, for it has been shown that estrogen administration to males or non-pregnant females produces a comparable rise in plasma transcortin concentration (87).

2. 17-ketosteroids.

From reports on measurements of 17-ketosteroid concentration in maternal and cord plasma, three pertinent facts emerge. First, the concentration of total 17-ketosteroids (91) as well as of dehydroepiandro-

sterone (92) is lower in the maternal circulation than in the foetal circulation.

Secondly, the concentration of dehydroepiandrosterone is significantly greater in the arterial plasma than in the venous plasma of the umbilical cord (93).

Finally, when foetal adrenal function is impaired, as in the case of the anencephalic foetus or, as a result of the administration of large doses of cortisone throughout pregnancy, the concentration of dehydroepiandrosterone in cord plasma is markedly reduced (93).

These observations indicate that there is a gradient in the concentration of 17-ketosteroids between the maternal and the foetal circulation, that 17-ketosteroids are being transfered from the foetus to the placenta and that the production of 17-ketosteroids by the foetus is dependent on the integrity of the foetal adrenal.

Yet in spite of this transfer from the foetus, the concentration of 17-ketosteroids in the maternal circulation remains exceedingly low. Indeed, according to Gardner (91) at term it is only one tenth that observed in the same woman prior to the onset of pregnancy. This is in keeping with the fact that, during pregnancy, the urinary excretion of dehydroepiandrosterone is reduced.

The logical conclusion is that 17-ketosteroids are "metabolized" by the placenta.

SUMMARY

There is no significant change in maternal adrenal structure during pregnancy. Minor differences in urinary 17-hydroxycorticosteroid and 17-ketosteroid excretion appear to be due to the presence of larger quantities of 17-hydroxyprogesterone metabolites in the urine. The

origin of 6β-hydroxysteroids in pregnancy urine has not been clearly defined.

Increased aldosterone and aldosterone metabolites in pregnancy urine reflect a higher secretion rate of this hormone. The estrogen excretion does not appear to be related to maternal adrenal function but rather to the biogenesis of such steroids in the foetoplacental unit. The elevated transcortin concentration in maternal plasma is likely due to the elevated level of circulating estrogens. Increased binding of cortisol to transcortin accounts for the high plasma 17-hydroxycorticosteroid concentration, and may also account for its prolonged half-life.

III. THE ROLE OF THE PLACENTA

A. STEROID SYNTHESIS

The studies of Diczfalusy (1) suggest that the placenta carries out little if any steroid biogenesis de novo but is dependent on the availability of precursors from the mother and the foetus.

1. Progesterone.

In the third trimester of pregnancy the placenta produces approximately 250 mg of progesterone daily (94,95). Since the interruption of the foetoplacental circulation, by ligation of the umbilical cord, causes only a slight reduction in the excretion of pregnanediol (96), it is assumed that placental progesterone arises chiefly from maternal precursors. Even though the source and the identity of these precursors has not been established, they do not appear to be derived from either the maternal ovaries or adrenals since neither oophorectomy nor adrenalectomy (97) affects pregnanediol excretion. Pertinent to this problem the conversion of cholesterol and pregnenolone to progesterone by placental tissue has been demonstrated in vitro (98) by perfusion of

term placenta (99), as well as the 17α-hydroxylation of progesterone by placental microsomes (100).

2. Estrogens.

The evidence suggesting the production of estrogens by the placenta is based chiefly on the isolation of relatively high concentrations of estrogens from the placenta (101), pregnancy urine (102), and blood (103), and also on the finding that these concentrations are not significantly reduced by the removal of maternal ovaries (104) adrenals (105) or pituitary (106) during the latter part of pregnancy.

Levitz has described the incorporation of ^{14}C acetate into ^{14}C estradiol in the perfused placenta but in subsequent studies could not confirm this finding (107).

The presence of enzyme systems capable of aromatizing neutral C-19 steroids into estrogens has been observed by in vitro incubation of placents mince (90) as well as in perfusion experiments (108). In contrast, it has not been possible to demonstrate the conversion of progesterone (through 17α-hydroxyprogesterone) into estrogens, implying that the placenta does not possess significant amounts of desmolase capable of side-chain cleavage (109). Casmer's experiments (96) show an abrupt drop in urinary estrogen excretion when the foetoplacental circulation is interrupted. These studies together with the clinical observations that a decrease in maternal urinary estrogens (more specifically, estriol) reflects foetal distress or foetal death (54) suggest that placental estrogen production is primarily dependent on a supply of precursors from the foetus.

In this context Bolté (51), in a series of three studies performed on pregnant women undergoing therapeutic abortion, has provided the critical evidence which establishes that in vivo the placenta is extremely active in the conversion of foetal androgens.

In the first study the foetus was removed and the placenta was perfused in situ. In the second study, dehydroepiandrosterone was injected into the uterine artery. In the third, the umbilical cord was delivered through the cervix, dehydroepiandrosterone injected into the umbilical vein and the maternal antecubital vein and the pregnancy was allowed to continue for six days. Rapid aromatization of dehydroepiandrosterone occurred during placental perfusion; estrone and estradiol were isolated from the placenta, the perfusate and from maternal urine, but estriol could not be detected. The injection of dehydroepiandrosterone into the uterine artery demonstrated that essentially the same aromatizing process was effective from the maternal side of the placenta, as had been observed following placental perfusion, and in both instances free dehydroepiandrosterone was aromatized more readily than its sulfate ester. Regardless of whether dehydroepiandrosterone or its sulfate ester was injected, 80% of the radioactive material recovered from the placenta appeared in the free steroid fraction, whereas over 80% of the radioactivity recovered from the foetus was present in the conjugated fraction. In the third study Bolté examined the overall contribution to urinary estrogens of dehydroepiandrosterone sulfate circulating in the foetal and maternal compartments. He found that 75% of the androgen sulfate injected into the foetal compartment was converted into estrogens, as compared to a 25% conversion when the steroid was injected into the maternal compartment. The formation of estriol by the foetus was considerably greater than by the mother.

Diczfalusy (1) and Bolté (51) proposed that the placental conversion of dehydroepiandrosterone to estrogens occurs via androstenedione and testosterone.

3. Adrenocorticoids.

There is very little experimental evidence to suggest that the

placenta produces adrenocorticosteroids. De Courcy (110) reported the isolation and identification of a number of steroids including cortisol, cortisone and aldosterone following the extraction of placental tissue. Studies of this type are complicated by the fact that the placenta contains a considerable amount of maternal and foetal blood which may account for 10% of placental weight (111). In view of the very high levels of free 17-hydroxycorticosteroids found in maternal plasma it seems reasonable to conclude that most of the hormones isolated following placental extraction might have been present in the blood trapped within the tissue.

Sylbuski (113) found the amount of corticosteroids present in the placenta prior to incubation was equal to the amount released into the media during in vitro incubation and that no additional steroids were formed by the placenta during the incubation period when precursors such as progesterone, cholesterol and pregnenolone were used.

Only Troen (112), utilizing an in vitro perfusion technique, reported the production of corticosteroids by the placenta, and found that half of these were glucuronide conjugates. These studies should be repeated, for the data they present is clearly in conflict with the results obtained by others.

Sylbuski reported the conversion of cortisol to cortisone in human placental mince (113) and Meigs (114) the interconversion of secondary hydroxyl and ketone functions in the 3 and 11 positions and reduction of the 20-ketone in slices and subcellular fractions of placenta. The report of Osinski (115) indicates the presence in placenta of 11β-hydroxydehydrogenase capable of converting cortisol, corticosterone and 11β-hydroxyandrostenedione to the corresponding 11-keto derivatives.

SUMMARY

The placenta is unable to form steroids from two carbon frag-

ments but can elaborate large amounts of estrogens and progesterone from precursors originating in the foetal or maternal circulation. This activity is centered around three main enzyme systems, namely: sulfatases, 3β-hydroxylase and aromatizing enzymes.

There is as yet no convincing evidence that the placenta produces appreciable amounts of corticosteroids, but it does appear to limit the transfer of cortisol from the maternal into the foetal circulation. It could be postulated that by this means foetal tissues are protected from the high concentration of this hormone in the maternal circulation.

The aromatization of androgens into phenolic estrogens could serve to protect the foetal tissues from androgen excess. For although dehydroepiandrosterone is itself a weak androgen, virilization of the foetus could presumably occur if testosterone and androstenedione, formed during dehydroepiandrosterone metabolism, were to accummulate.

IV. ADRENOCORTICAL FUNCTION AND STEROID METABOLISM OF THE NEWBORN INFANT

Differences in plasma and urinary corticosteroids, and in the response to ACTH and stress suggest that the adrenal function and corticosteroid metabolism of the normal newborn may differ significantly from that of the adult.

A. PLASMA CORTICOSTEROIDS

To evaluate reports of plasma corticosteroids in the newborn period, it might be better to consider studies on cord plasma separately from those performed on peripheral plasma during the first days of life. The interpretation of the first group of studies is difficult because of the complex metabolic and endocrine inter-relationship of the maternal-foetal-placental unit. The second type of studies reflects more directly the autonomous adrenal function of the newborn infant.

1. Cord plasma.

The concentrations of 17-hydroxycorticosteroids measured by the Porter-Silber reaction in mixed arterial and venous plasma from the umbilical cord are higher than those observed in plasma of normal adults (116-121). Although a wide range of values is reported (16-55 µg/100 ml) the majority lie between 20 and 25 µg/100 ml.

These studies have been criticized by Ulstrom (123) who found in the cord and in the peripheral plasma of the newborn, relatively high concentration of non-specific chromogens and unidentified Porter-Silber-positive steroids other than cortisol. The same author reported that the concentration of cortisol in cord blood, when measured by isotope dilution, was 8 µg%, a value not significantly different from that of the adult male or non-pregnant female (124).

The ratio of maternal to foetal plasma 17-hydroxycorticosteroids is between 5:1 and 2:1 (116,117). This difference in the distribution of 17-hydroxycorticosteroids across the placenta is confirmed by the finding that following administration of a tracer dose of ^{14}C cortisol at the time of delivery, the concentration of radioactivity in maternal plasma is three times greater than in cord plasma (125).

From these investigations, Migeon (83) introduced the concept of a "placental barrier" establishing a 17-hydroxycorticosteroid gradient between the maternal and foetal circulation. The 17-hydroxycorticosteroid levels in the latter circulation are thus considered to be a mere reflection of their concentration in maternal plasma. Migeon suggested that the underlying cause for this may be the low titer of transcortin in foetal plasma (85-87).

A simple diffusion of steroids across the placenta does not however explain the concentration of cortisone, which in cord plasma is three to four times greater than in maternal plasma (126,127).

The level of 17-ketosteroids and more specifically dehydroepiandrosterone and androsterone are also higher in cord than in maternal plasma (91,92). On the other hand low values of dehydroepiandrosterone are found in the cord plasma of anencephalic infants and in that of infants born to mothers treated with large doses of cortisone (93). These studies imply that the foetal adrenal is responsible for the high concentration of 17-ketosteroids in cord blood. Further evidence regarding the contribution of the foetal adrenal to the steroids found in the cord is provided by the studies of Eberlein (129,130), who isolated from pooled cord plasma more than twenty steroids of the C-19 and C-21 series, most of which possessed the 3β-hydroxy-$\Delta 5$ configuration.

2. Peripheral plasma of the newborn infant.

Studies reporting the measurement of plasma corticosteroid concentration in newborn infants present conflicting results. Klein (131), Ulstrom (123) and Gemzell (117) have indicated that following birth the concentration of free 17-hydroxycorticosteroid s falls to extremely low levels and remains below normal for the first month of life. More recently, Bertrand (118) reported that the newborn's plasma 17-hydroxycorticosteroids did not fall appreciably below normal values for older children. Ulstrom (123) in pooled plasma samples obtained during the first four days of life found that cortisol concentration rose from 8 µg% at birth to 16 µg% between the third and the twelfth hour of life, decreased from the twelfth to the thirty-sixth hour, and increased slightly during the subsequent two and a half days.

Seely (127) studied the quantitative and qualitative pattern of corticosteroids in pooled plasma samples from newborn infants. In infants 46 to 96 hours of age, the concentration of individual steroids - cortisol, cortisone, corticosterone and 11-dehydrocorticosterone - was lower than at birth, and as in cord plasma the concentration of cortisone and 11-dehydrocorticosterone was higher than that of the respective 11-hydroxylated compounds. In prepubertal children, males, and non-pregnant females, Seely (127) found the level of the former two steroids extremely low.

Plasma 17-ketosteroids fall rapidly during the first few days of life and dehydroepiandrosterone and androsterone, present in high concentration in cord plasma, cannot be detected in the plasma of the newborn 48 hours after birth (132).

In the newborn infant the concentration of transcortin is very low and is not increased by estrogen administration (122). The possibility that the formation of transcortin is deficient in early infancy is consistent with the observations of Hitzig (133) that this group of serum proteins are poorly synthesized during the first six months of life.

Bertrand's study (122) indicates that in the normal adult 60% of the circulating 17-hydroxycorticosteroids are present in the free form. During pregnancy, the level of non-conjugated 17-hydroxycorticosteroids increases while the conjugated fraction remains constant. At delivery, approximately 85% of the steroids in maternal as well as in cord plasma are unconjugated. During the first week of life there occurs a rapid increase in the conjugated fraction, and by one month of age more than 50% of the total circulating plasma 17-hydroxycorticosteroids is present in the conjugated fraction.

B. CORTISOL PRODUCTION RATE

The rate of cortisol production by newborn fullterm and premature infants has been reported by Kenny (134,135) and Bertrand (122). When corrected for surface area all the values fell within the normal range for adults (12 mg/m^2/day). In Kenny's study the highest average values were observed in infants less than five days of age (18.3 mg/m^2/day). The average production rate of infants over five days was slightly less (13.5 mg/m^2/day). The validity of the method used by Kenny and Bertrand is dependent on the existance of a steady state and uniform distribution of the injected ^{14}C cortisol (136). It is not positively established whether either one of these criteria is fulfilled in the newborn infants, therefore the results obtained require confirmation.

C. HALF-LIFE OF CORTISOL

In the adult it is generally accepted that the half-life of a tracer dose of cortisol is between 80 and 90 minutes (137-139). In the neonatal period, however, several investigations have led to the conclusion that the half-life of this steroid is prolonged. Bongiovanni (140) found that in nine babies given 25 mg of cortisol hemisuccinate on the first day of life the mean half-life established from measurements of 17-hydroxycorticosteroids was 997 minutes. Grumbach (141), using a dose of 7-10 mg/kg reported a mean half-life of 220 minutes, whereas that of cortisol-free alcohol given in a dose of 4 mg/kg was 126 minutes. Reynolds (142) gave 5 mg/kg of cortisol free alcohol to infants 32 to 108 hours of age and observed a half-life of 255 minutes for 17-hydroxycorticosteroids and 204 minutes for cortisol. Cranny (143) gave 1 mg/kg of cortisol free alcohol to three babies 2, 3 and 4 days of age, and found the half-life of the steroid to be 86, 150 and 77 minutes respectively.

The significance of studies using large amounts of cortisol is questionable. The values reported appear to be related to the injected dose. Samuels (144) has shown that the rate of metabolism of a given steroid is affected by the amount injected. In the newborn, with limited cortisol binding capacity and a possible limitation of overall hepatic function, the dose of steroid administered may be even more critical. In this respect, the studies of Seely (145) are of particular interest, for he has shown that following the administration of a tracer dose of ^{14}C cortisol the half life of the total radioactivity was 245 minutes, whereas that of free cortisol was only 44 minutes. (Seely observed a rapid conversion of cortisol to cortisone: 30 minutes after injection the concentration of labelled cortisone exceeded that of cortisol.)

The rapid disappearance of cortisol from the plasma of the newborn infant which is indicated by this latter report, is similar to that described in 5 to 6-month foetuses. Leyssac (146) measured simultaneously the cortisol concentration in maternal, cord and foetal heart plasma during therapeutic abortions. When cortisol was administered at a constant rate during the operation its plasma concentration in the foetal heart was 30% lower than in the umbilical cord. From this observation it was concluded that in the foetus cortisol metabolism was very rapid.

D. ACTH STIMULATION

Most of the studies on the measurement of adrenocortical steroids in the urine of the newborn infant have suggested that there exists a state of hypofunction (147-151) and relative unresponsiveness to stress or ACTH administration during the first days of life (152,153).

Salmi (154) however found that the urinary 17-hydroxycorticosteroid excretion of the newborn infant, when corrected to a standard surface area, was increased following ACTH stimulation to a level corresponding to the amounts found in adults after major stress. Expressing his results in the same way, Cranny (155) reported steroid excretion of normal fullterm babies to be comparable to adult excretion values. However in the presence of prematurity or respiratory distress the 17-hydroxycorticosteroid excretion was extremely low, suggesting adrenal insufficiency (155).

The interpretation of these results is extremely difficult for, as Colle (153) has indicated, the standard procedures of 17-hydroxycorticosteroid extraction will not lead to the detection of 6β-hydroxysteroids which are major cortisol metabolites in newborn urine.

Klein and Rovnanek (156) showed that the 3-day-old infant responds to ACTH administration by an increase of free plasma 17-hydroxycorticosteroids comparable to that observed in adults.

Bertrand (118) observed normal free plasma 17-hydroxycorticosteroid response to ACTH injection in the first hours of life.

E. URINARY CORTISOL METABOLITES

Bertrand (122) following the administration of ^{14}C cortisol to newborn infants reported that of the total urinary radioactivity 10% was present in the free fraction, 15% in the glucuronide fraction and 21% was subsequently realized by acid hydrolysis. 54% remained unextractable. The corresponding values in adults were: free fraction, 7%; glucuronide fraction, 46%; acid hydrolysis, 7%; unextractable, 40%. Thus it appears that the urine of the newborn infant contains considerably less glucuronide and a greater amount of cortisol metabolites realized by acid hydrolysis. In spite of slightly different methodological approaches the studies of Kenny (134), Seely (145), Aarskog (157), Reynolds (158) and Migeon (147) are in general agreement with Bertrand's findings.

The decreased amounts of steroid glucuronides found in the urine of newborn infants may be due to one or several factors: Bertrand has suggested that the apparent defect in glucuronide formation could be related to the prolonged exposure of the foetus to a high concentration of progesterone (122) since Hsia has shown, in vitro, that this steroid inhibits glucuronosyl transferase (159).

Alternate pathways of corticosteroid catabolism in the newborn infant might also lead to a reduction of the quantity of steroids excreted as glucuronides. Ulstrom (160) has suggested that the large quantities

of 6β-hydroxycortisol found in the free fraction of newborn urine need not be interpreted as a failure of glucuronide formation but rather might reflect the fact that the compound is either not suitable for or does not require conjugation.

The importance of ester sulfates as an alternate mode of steroid conjugation in the newborn period was suggested by Drayer who found that the 17-hydroxycorticosteroid content of the "sulfate" fraction was as great or greater than that of the free or β-glucuronidase hydrolyzed fractions (161).

Based on his observation that the half-life of cortisol (see above) was prolonged in the newborn infant whereas that of tetrahydrocortisol was normal, Bongiovanni has proposed that reduction of the steroid molecule at the level of the Δ^4-3 ketone group is impaired (164). This would result in a decreased formation of tetrahydro metabolites which in adults are excreted as glucuronides.

The above theories cannot be adequately assessed without knowing more precisely the nature and relative proportions of individual steroids in the different fractions of newborn infants' urine. Such systematic studies are not available.

Cathro (70) reported that tetrahydrocortisol and tetrahydrocortisone were excreted in very small amounts by the newborn and confirmed Ulstrom's observations of an increased concentration of 6β-hydroxycortisol (160). Cathro suggested that the presence of this compound may reflect an alteration in normal cortisol metabolism as a direct effect of estrogens. The main feature in the analysis of the urinary C-21 steroids was the predominance of blue tetrazolium-reducing compounds, different from those found in adult urine in all ranges of polarity. The 11-desoxy and 11-oxygenated 17-keto-

steroid fraction contained practically none of the 17-ketosteroids normally found in the adult, however this fraction did contain large amounts of Zimmerman-reacting substances that could be readily distinguished chromatographically from the known 17-ketosteroids.

SUMMARY

One can conclude that a passive transfer of steroids from the maternal circulation is not the only factor determining the concentration of corticosteroids in cord blood. Recent evidence leaves little doubt that the foetal adrenal produces a number of steroids, some of which the placenta utilizes as estrogen precursors. Among these compounds there appears to be a predominance of steroids of the 3β-hydroxy-Δ^5 configuration. The presence of these compounds in cord blood is further evidence that 3β-hydroxysteroid dehydrogenase activity is markedly reduced in foetal adrenal tissue. However, the normal cortisol production rate, the normal concentration of cortisol in the peripheral plasma of the newborn infant, and its adequate response to ACTH stimulation indicate that the activity of the enzyme is present immediately after birth.

In the blood and urine of newborn infants the amount of cortisol metabolites present as glucuronides is definitely lower than in the normal adult. More quantitative and qualitative data are needed to clarify whether this lesser proportion of steroid glucuronides is related to the formation of metbolites of cortisol which do not require conjugation,or to the formation of conjugates other than glucuronides, or to both.

EXPERIMENTAL STUDIES

I. PURPOSE OF EXPERIMENTAL STUDIES

The experimental section of this thesis is presented under three main headings:

1. In vitro studies of the human newborn adrenal cortex.
2. Plasma cortisone and cortisol levels at birth and during the neonatal period.
3. In vitro studies of 11β-hydroxydehydrogenase activity in human placenta.

At the time the studies were initiated the purpose of these experiments was, firstly, to investigate whether at birth the foetal adrenal, and in particular the foetal zone, is capable of forming biologically active C-21 steroids. Secondly, it was felt that previous reports concerning the distribution of 17-hydroxycorticosteroids in maternal and cord plasma needed to be reinvestigated. A double isotope derivative assay was developed which measured cortisol specifically. Utilizing this technique, it was possible to follow the concentration of cortisol during the first few days of life and to study its response to ACTH stimulation.

In the course of these later investigations cortisone was found in relatively high concentrations in maternal, cord, and in the plasma of the newborn infant. It was reasonable to assume that the presence of cortisone at least in the cord might be due to the dehydrogenation of cortisol during transplacental transfer of this steroid, therefore an attempt was made to gain further insight into the kinetics of 11β-hydroxydehydrogenase activity of placental microsomes.

The methods, results, and discussion pertinent to each of the

three studies will be presented in separate chapters. An attempt will be made to correlate the results of these investigations in a general discussion.

II. IN VITRO STUDIES OF THE HUMAN NEWBORN ADRENAL CORTEX

In vitro, the biosynthetic capacity of the foetal adrenal has previously been assessed by incubation of adrenals from foetuses obtained at the time of therapeutic abortion during the first half of pregnancy. These reports supported the earlier concept that the foetal adrenal has a predominantly androgenic function. Villee (45) suggests that foetal development is accompanied by a process of enzymatic maturation which changes the pattern of foetal adrenocorticoid biogenesis. Bloch (37) noted that the ratio of extractable C-19 steroids to cortisol and cortisone decreased from 100:1 at the 10th week of gestation to 7:1 by the 20th week.

The following study reports observations on the incubation of adrenals from premature and full term infants in the presence of ^{14}C-progesterone. The results suggest that from this precursor the biogenetic capacity of both the adult cortex and the foetal zone are mainly directed towards the production of C-21 steroids from the 22nd to the 38th week of gestation. In addition it was demonstrated that in the absence of added precursor both the adult and the foetal zone produced cortisol and that the production of this steroid was stimulated by ACTH.

In contrast to earlier studies which have attempted to define the steroidogenic capacity of the foetal adrenal during pregnancy, the results of these experiments reflect the in vitro biogenetic activity of the adrenal at birth and therefore may bear a more direct relationship to neonatal adrenal function.

A. METHODS

Adrenals were obtained from two full term and nine premature infants dying within the first three days of life. Death was attributed to prematurity and/or the respiratory distress syndrome (162). Urine was collected continuously up to the time of death from three of these premature infants who survived 23, 60 and 70 hours. The total urinary 17-hydroxycorticosteroid excretion measured by the method of Silber-Porter (163) in these infants and in thirteen infants who survived severe respiratory distress was compared to that of eight normal infants (Table I). The values obtained were significantly higher ($P<0.05$) in infants with respiratory distress (68 µg/kg/day) than in normal newborns (33 µg/kg/day). Steroid excretion in premature and full term infants when corrected for differences in body weight were comparable and the elevation of total 17-hydroxycorticosteroids in infants with respiratory distress did not appear to be related to the severity of the disease.

All adrenals were obtained within four hours of death. The methods of dissection and incubation of the tissue, extraction of the incubation media and preliminary purification of these extracts by paper chromatography were very similar to those previously described by Stachenko and Giroud (165). The glands were freed of peripheral fat and cut into slices 0.3 - 0.5 mm thick. No attempt was made to remove the medulla which, in this age group, represents a negligible proportion of the whole adrenal. The slices were then divided into the "foetal" and "adult" cortex under a dissecting microscope (x 10). Histologically it may be seen (Figures 1 and 2) that such a procedure yields two distinctive kinds of tissue: that obtained from the outer third of the gland, which is composed of the connective tissue of the capsule, the adult cortex and a variable proportion of the foetal zone; and that

TABLE I

URINARY 17-HYDROXYCORTICOSTEROID EXCRETION IN NORMAL NEWBORNS AND INFANTS WITH THE "RESPIRATORY DISTRESS SYNDROME" DURING THE FIRST THREE DAYS OF LIFE

	NUMBER OF INFANTS	17-HYDROXYCORTICOSTEROIDS(μg/Kg/Day)					
		1st DAY		2nd DAY		3rd DAY	
		FREE / CONJUGATE	TOTAL	FREE / CONJUGATE	TOTAL	FREE / CONJUGATE	TOTAL
NORMAL	8	8.9 / 11.4	20.3	19.2 / 21.7	40.9	18.4 / 21.7	40.1
RESPIRATORY DISTRESS	16	14.5 / 25.5	40.0	40 / 46	88.0	30.3 / 45.5	75.5
p VALUE			< 0.05		< 0.01		< 0.01

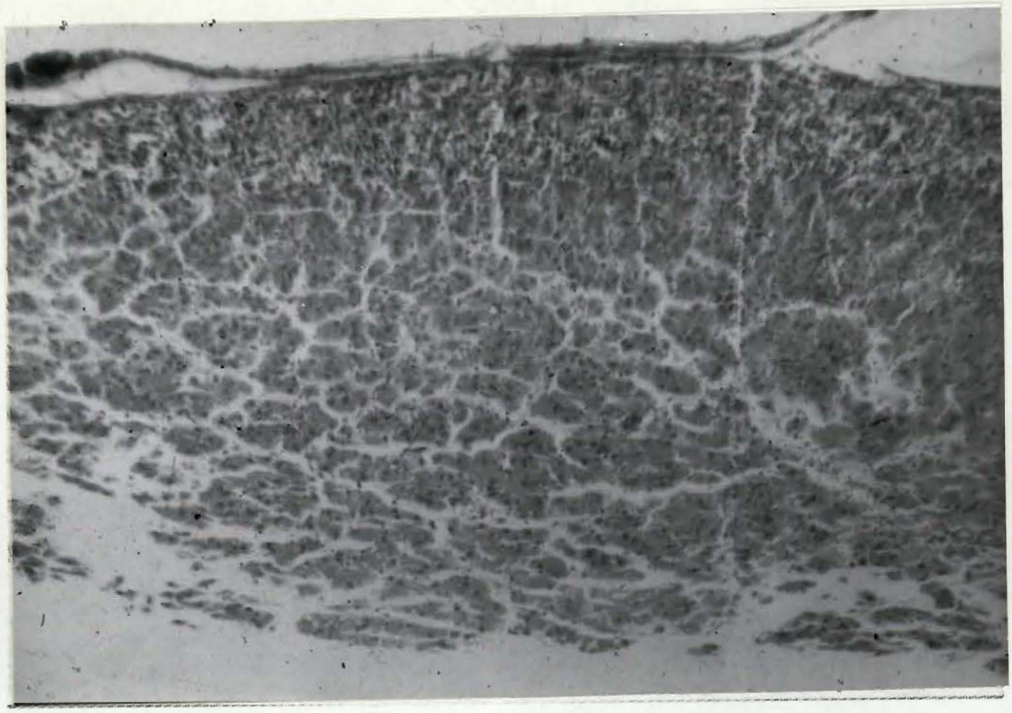


Figure 1. ADULT CORTEX OF THE ADRENAL OF A FULL TERM INFANT. THE CAPSULE OF THE GLAND IS AT THE TOP OF THE FIGURE. BELOW ARE THE SMALL TIGHTLY PACKED CELLS OF THE ADULT CORTEX AND A LARGE PORTION OF THE FOETAL ZONE.

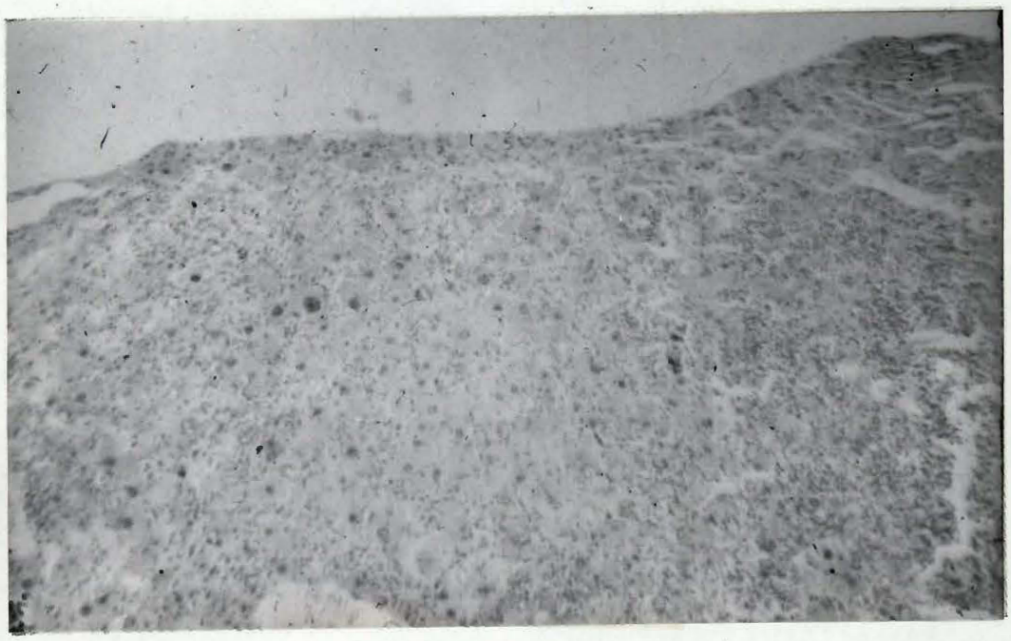


Figure 2. FOETAL ZONE OF THE ADRENAL OF A FULL TERM INFANT. THE SECTION CONSISTS ENTIRELY OF LARGE LOOSELY ARRANGED FOETAL ZONE CELLS.

obtained from the inner two-thirds, which is almost entirely made up of foetal cortex, occasionally showing discrete areas of necrosis. Each group of slices was further divided into two portions of equal weight. From the time the adrenals were removed until the beginning of the incubation, an average period of 1.5 hours, the tissues were kept at 5 to 8° C. in 0.9% sodium chloride, except for the time necessary to prepare individual slices which were dissected on filter paper, repeatedly moistened with the same solution. Each group of slices was then put into 50 ml beakers containing 10 ml of Krebs-Ringer bicarbonate medium with added glucose (200 mg%) per gram of tissue. The incubations were performed in a Dubnoff Metabolic Shaker Incubator in an atmosphere of O_2-5% CO_2, at 37 ± 1° C. The flow of gas was regulated at 7 liters/minute and the rate of shaking at about 100 movements/minute. The slices were preincubated for 30 minutes (166), after which the media were renewed and the incubation allowed to proceed for 90 minutes. At the beginning of this latter period 5 I.U. of ACTH (Nordic Biochemicals, Lot no. A8501) were added to the incubation media of one group of "adult" and one group of foetal zone slices, the second group serving as control. In the case of the control groups, the media were again renewed at the end of this period and the incubation continued for 3 hours in the presence of ^{14}C progesterone. Except if otherwise indicated this precursor was added at the dose level of 100 µg/g. of tissue incubated. Its specific activity was equal to 5.2 µc/mg. At the end of the incubation the media were decanted and frozen until extraction.

Solvents used for extraction and chromatography were redistilled

once in an all glass apparatus fitted with a Vigreux column.

The media were extracted first with twice and then with once their volume of chilled chloroform. The chloroform extracts were filtered into round-bottom flasks and taken to dryness under vacuum.

In the absence of added precursor the individual glands produced such small amounts of steroids that only an approximation of the material produced could be obtained by spectrophotometric measurement at 240 mμ of the whole crude extracts. After two chromatographic separations the attendant loss further reduced the quantities available for measurements so that it was impossible to characterize properly any of these steroids. Consequently, in two experiments, the residues of the incubation media to which no precursor had been added were pooled for further study.

The extracts of the incubation media to which radioactive progesterone had been added were first developed for 9 hours in the B_5 system of Bush (164) - benzene: methanol: water (2:1:1). The cortisol and corticosterone fractions were eluted with methanol, reapplied and developed for 8 hours in the E_2B system described by Eberlein and Bongiovanni (176) - Iso-octane: tert-butanol: water (5:2.5:4.5). The specific activities of cortisol and corticosterone reported below for individual glands were established after elution from this second chromatogram. The solvent front of the first chromatogram was eluted, reapplied and developed for 6 hours in the B_3 system of Bush (164) - light petroleum: benzene: methanol: water (667:333:600:400) in which small radioactive areas corresponding to the chromatographic mobilities of the reference standards of 17 -hydroxyprogesterone and desoxycorticosterone were separated. The solvent front of this chromatogram

developed in the Bush A system (164) led in 6 hours to the resolution of a single band with the chromatographic mobility of progesterone.

Partial identification of ^{14}C steroid fractions was attempted following addition of non-radioactive carriers (167) and sequential preparation of acetate and chromic acid oxidation derivatives (167, 168). After further chromatography of these derivatives in appropriate systems, the observation of constant specific activity was interpreted as a criterion of identity with the added carrier.

All counts were obtained under a Nuclear Chicago "micromil end-window" gas flow counter operated in the Geiger range. In every instance an infinitely thin sample (no more than 20 µg of steroid), was plated, and the number of counts recorded was sufficient to ensure a precision of at least ± 5%. The radiochemical purity of ^{14}C-progesterone was checked by chromatography.

Quantitative determinations of individual Δ^4-3 ketosteroids were performed in a Unicam spectrophotometer either by the maximal absorption at 240 mµ using the Allen correction factor (166), or by the isonicotinic acid hydrazide reaction (170). In certain experiments the cortisol fraction was measured by the Porter-Silber reaction (171).

Radiochromatograms were scanned under a windowless gas flow counter (Nuclear Chicago - Actigraph II).

B. RESULTS

1. Production of steroids by the adult and foetal cortex in the presence of ^{14}C-progesterone.

a) Qualitative results.

Direct examination or contact photography under ultraviolet light of the paper chromatograms developed in the B_5 system showed in every instance two areas of ultraviolet absorbing material

possessing a chromatographic mobility similar to that of cortisol and corticosterone respectively (Figure 3). They were always of greater intensity on the strips relating to the adult cortex. In contrast, after elution of the solvent front, reapplication and development in the B_3 system, no detectable U.V. absorbing bands were observed with the exception of one, close to the front itself. Its chromatographic mobility was that of progesterone.

The radioactive scan of a typical chromatogram developed in the B_5 system (Figure 5) indicates a qualitatively similar distribution of radioactivity in the cortisol and corticosterone fractions produced from the adult and foetal cortical slices. After elution of the corticosterone fraction and second chromatography in the E_2B system,a definite peak of radioactivity in the area coinciding with the reference standard of 11-desoxycortisol separated from the bulk of corticosterone, whereas the cortisol fraction behaved as an homogeneous Δ^4-3-ketosteroid presenting a single peak of radioactivity. On rechromatography in the B_3 system of the material less polar than the corticosterone fraction on the B_5 system, at least four distinct areas of radioactivity were detected on the scanning. These corresponded to the mobility of the reference standards of 11-dehydrocorticosterone, 17α-hydroxyprogesterone, desoxycorticosterone and progesterone.

b) Partial characterization of steroids by derivative formation.

After elution of the cortisol and corticosterone fractions from the second chromatogram, 40 - 50 µg of pure non-radioactive carriers were added and the specific activities were determined. These

Figure 3 Figure 4

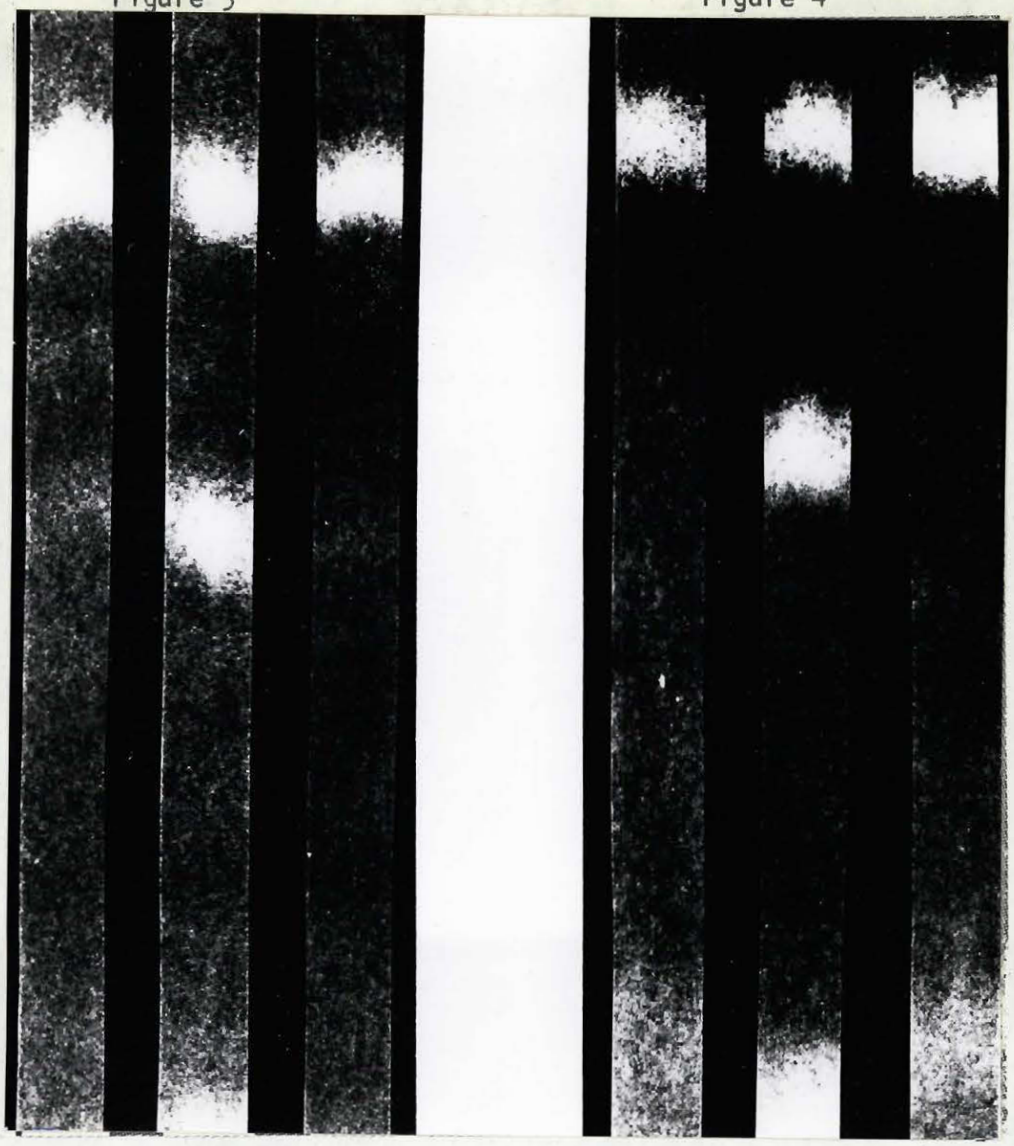


FIG. 3. U.V. PHOTOSTAT OF A CHROMATOGRAM DEVELOPED IN THE BUSH B5 SYSTEM. PREMATURE INFANT, GESTATIONAL AGE 32 WEEKS. *MIDDLE STRIP* - REFERENCE PILOTS; FROM TOP TO BOTTOM: CORTISOL, CORTICOSTERONE (10 μg. each). *LEFT STRIP* - EXTRACT OF THE INCUBATION MEDIA OF 500 MG. OF 'ADULT' CORTEX. *RIGHT STRIP* - EXTRACT OF THE INCUBATION MEDIA OF 590 MG. OF FOETAL ZONE.

FIG. 4 U.V. PHOTOSTAT OF A CHROMATOGRAM DEVELOPED IN THE BUSH B5 SYSTEM. *MIDDLE STRIP* - REFERENCE PILOTS; FROM TOP TO BOTTOM: CORTISOL, CORTISONE, CORTICOSTERONE (10 μg. each). *RIGHT STRIP* - EXTRACT OF THE INCUBATION MEDIA OF 856 MG. OF WHOLE ADRENAL SLICES FROM THREE PREMATURE INFANTS (22 WEEK'S GESTATION) INCUBATED IN THE PRESENCE OF ACTH. *LEFT STRIP* - CONTROL. IDENTICAL WEIGHT OF TISSUE.

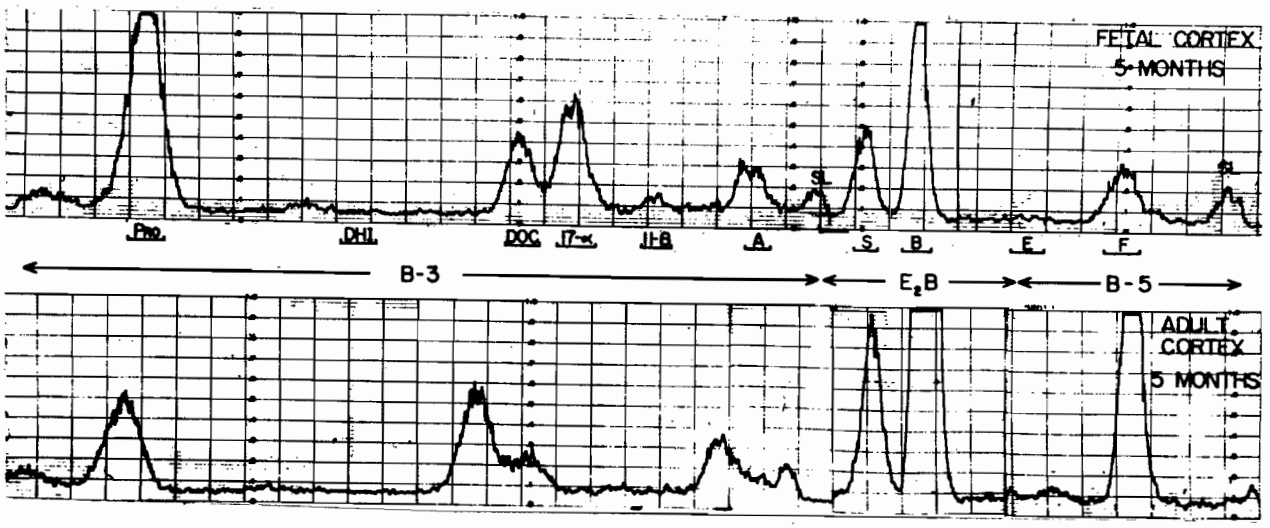


FIG. 5. STUDIES OF THE IN-VITRO PRODUCTION OF CORTICOSTEROIDS BY SLICES OF 'ADULT' CORTEX AND FOETAL ZONE. TWIN NEWBORN INFANTS, GESTATIONAL AGE 22 WEEKS. INCUBATION PERFORMED IN THE PRESENCE OF ^{14}C-PROGESTERONE. THE FIGURE REPRESENTS THE SCANNING OF THE RADIOCHROMATOGRAMS DEVELOPED IN THE SYSTEMS INDICATED. FOR FURTHER INFORMATION REFER TO TEXT.
UPPER HALF - 350 MG. OF FOETAL CORTEX. *LOWER HALF* - 500 MG. OF 'ADULT' CORTEX.
COUNT RATE SETTING - 1000 C.P.M. *SLIT WIDTH* - $\frac{1}{8}$IN.

SL : STARTING LINE; F : CORTISOL; E : CORTISONE; B : CORTICOSTERONE; S : 11-DEOXYCORTISOL; A : 11-DEHYDROCORTICOSTERONE; 11β : 11β-HYDROXYANDROSTENEDIONE; 17α : 17α-HYDROXYPROGESTERONE; DOC : DESOXYCORTICOSTERONE; DHI : DEHYDROEPIANDROSTERONE; PRO : PROGESTERONE.

fractions were then acetylated with acetic anhydride in pyridine and the product of acetylation chromatographed in the B_3 system. Their mobilities were identical to that of the reference standards of cortisol acetate and corticosterone acetate respectively. The specific activities of the acetate derivatives were determined. A second derivative was then prepared by subjecting the acetates to oxidation by chromium trioxide in glacial acetic acid. The resulting acetate of the 11-dehydro derivatives were separated by chromatography in the B_3 system. Their mobilities corresponded to that of the reference standards of cortisone acetate and 11-dehydrocorticosterone acetate respectively. Their specific activities were established. It can be seen (Table II) that by this series of tests an acceptable radiochemical purity could be achieved for cortisol and corticosterone.

In a single instance the 11-desoxycortisol fraction eluted from the E_2B chromatogram was diluted with 30 μg of 11-desoxycortisol carrier. The specific activity was 360 c.p.m./μg. The fraction was acetylated and chromatographed in the B_3 system. The specific activity of the acetate was found to be 349 c.p.m./μg. Upon similar dilution with 17α-hydroxyprogesterone and desoxycorticosterone carriers, the radioactive areas coinciding with the mobility of these steroids in the Bush B_3 had an initial specific activity of 180 c.p.m./μg for 17α-hydroxyprogesterone and 94 cp.m./μg for desoxycorticosterone. The acetate of the desoxycorticosterone fraction counted 82 c.p.m./μg. Following acetylation and second chromatography in the system B_3 of Bush the mobility of the 17α-hydroxyprogesterone fraction remained unchanged, its specific activity being 174 c.p.m./μg. No attempt was made to study the radioactive material possessing the chromatographic mobility

TABLE II

CHARACTERIZATION OF CORTISOL AND CORTICOSTERONE BY CARRIER DILUTION AND DERIVATIVE FORMATION

	SPECIFIC ACTIVITY COUNTS / MINUTE / MICROGRAM					
TYPE OF TISSUE	CORTISOL (1)	CORTISOL ACETATE	CORTISONE ACETATE (2)	CORTICOSTERONE (1)	CORTICOSTERONE ACETATE	11-DEHYDROCORTICOSTERONE (3) ACETATE
Whole Cortical Slices	2780	2660	2730	2180	2410	2530
Foetal Cortex	120	120	112	78	65	62
Adult Cortex	350	333	313	505	490	493

(1) The first specific activity (S.A.) was obtained after two chromatographies in the systems B_5 of Bush and E_2B of Eberlein Bongiovanni. The S.A. of cortisol and corticosterone produced by whole cortical slices were established without carrier dilution.

(2) CrO_3 oxidation derivative of cortisol acetate.

(3) CrO_3 oxidation derivative of corticosterone acetate.

of 11-dehydrocorticosterone in the B_3 system.

c) Quantitative results.

Six pairs of adrenals were obtained from 5 prematures and 1 full term newborn (Table III). The foetal age of the prematures ranged between 22 and 36 weeks. Within this series in only two instances did death occur after more than 24 hours following delivery. In each study the foetal zone was separated from the adult cortex. The weight of the two portions of the glands are given in the table. Although special care was taken to separate the outer third from the inner two-thirds of the gland it will be noted that in three instances the part of tissue designated as adult cortex was of greater weight than that of the foetal zone. Since the foetal zone occupies 80% of the whole cortex it is clear that in all instances the adult cortex was contaminated by the foetal zone (Figure 1). It is also conceivable that the extreme friability of the foetal zone resulted in loss of tissue mass during the process of slicing and blotting of slices on filter paper in order to remove excess of saline.

The tissue was incubated for 3 hours in the presence of ^{14}C-progesterone (100 µg/gm of tissue; 2600 c.p.m./µg). In almost every experiment it was possible after two chromatographies to assess the specific activity of the cortisol and corticosterone produced by both the foetal and adult cortical slices. These results are presented in Table III. In spite of variations which cannot be correlated either with the foetal age or the time which elapsed between delivery and death, the data indicate that the foetal as well as the adult cortex is able to metabolize progesterone to cortisol and corticosterone

TABLE III

PRODUCTION OF CORTISOL AND CORTICOSTERONE BY THE ADULT AND FETAL CORTEX INCUBATED IN THE PRESENCE OF 4^{14}-C PROGESTERONE (1)

FETAL AGE : Weeks	POST-NATAL AGE : Hours	BIRTH WEIGHT: Gm.	WEIGHT OF TISSUE : mg.		µg/Gm of tissue/3 hrs				SPECIFIC ACTIVITY counts / minute / µg			
					CORTISOL		CORTICOSTERONE		CORTISOL		CORTICOSTERONE	
					A*	F +	A	F	A	F	A	F
38	23	2800	A*	1956	10.0		9.2		1080		2043	
			F	2400		2.0		4.0		1860		2110
36	23	1640	A	1140	4.2		5.0		987		1740	
			F	1642		3.0		5.0		1460		1162
32	23	1360	A	500	12.0		8.0		1023		2500	
			F	590		3.0		–		1594		–
30	70	690	A	710	15.0		14.0		2410		1190	
			F	544		5.0		11.0		2360		707
25	60	620	A	630	20.0		19.4		930		1125	
			F	320		–		–		–		–
22	4	412	A	500	9.0		12.4		2280		2145	
			F	330		2.0		2.4		1880		–

(1) 10 µg/100 mg. of tissue. Specific activity: 2600 c.p.m./µg

* A = Adult Cortex

\+ F = Foetal Cortex

and therefore imply the presence of 17, 21 and 11-hydroxylase activities in these two types of tissues from the 22nd week of gestation to term. They also suggest that in this respect the biogenetic capacity of the adult cortex is greater than that of the foetal zone. It is difficult to decide whether this reflects the rapid involution of the foetal zone which is known to occur after birth, irrespective of gestational age; or a limitation of this tissue to produce C-21 steroids.

In the last two columns of Table II are presented the specific activities (expressed in c.p.m./µg) of the cortisol and corticosterone produced. From the specific activity of the added ^{14}C-progesterone and the molecular weight of the progesterone, cortisol and corticosterone, the production of cortisol and corticosterone by the gland itself can be estimated. The values of steroid production calculated in this way ranged from negligible amounts to 12 µg for cortisol and 7.7 µg/g./3hr. for the corticosterone produced by the adult cortex, and a maximum of 1.1 µg for the cortisol and 7.7 µg/g./3hr. for the corticosterone produced by the foetal zone. Here again, despite the large variations between the different glands, the results of individual incubations suggest a lower internal production of cortisol by the foetal zone than by the adult cortex.

2. Action of ACTH on the production of steroids by the adult and foetal cortex.

The amount of material possessing the chromatographic mobility of cortisol and corticosterone produced by the foetal and adult cortex in the absence of added precursor was too small to quantitate after

proper chromatographic separation. Therefore the effect of ACTH on the production of these steroids by individual glands could not be assessed. However, measurements of total Δ^4-3 ketosteroids in the extracts of the incubation media indicated that ACTH increased the production of these compounds and that this effect was more apparent in the extracts of the adult cortex (Table IV).

In an attempt to study the effect of ACTH on the production of cortisol, the crude extracts of the incubation media of the foetal zone and of the adult cortex of four adrenals obtained from 3 premature infants (gestational age: 6 - 8 months) and 1 full term newborn, were pooled and submitted to paper chromatography in the B_5 system. The weights of the control and ACTH stimulated slices of the adult cortex were equal to 2.4 g. - those of the two groups of slices obtained from the foetal cortex to 2.5 g. The material eluted from the cortisol region after this single chromatography was measured by the Porter-Silber reaction (0.12 ml of ethanol and 0.18 ml of phenyl hydrazine sulfuric acid reagent). In every instance a symmetrical peak of absorption with a characteristic absorption maximum at 410 mμ was obtained between 370 and 450 mμ. The results indicate that the amount of this material was increased in both the foetal zone and the adult cortex stimulated by ACTH for a period of 90 minutes. The amount of cortisol produced by the adult cortex increased from 12.6 to 23.0 μg/100 g./hr. as a result of ACTH stimulation. The amount produced by the foetal zone increased from a control value of 3.6 to 6.6 μg/100 g./hr.

In a further attempt to determine the responsiveness of the newborn adrenal to ACTH stimulation whole adrenal slices from 3 prematures of 22 weeks gestation were incubated in the presence of ^{14}C-progesterone (2.5 μg/100 mg of tissue; specific activity: 2600 c.p.m./μg). Two por-

TABLE IV

EFFECT OF ACTH ON THE PRODUCTION OF " Δ^{4}-3 KETOSTEROIDS"
BY THE ADULT AND FETAL CORTEX

FETAL AGE : WEEKS	WEIGHT OF TISSUE : mg		µg EQUIVALENT OF CORTISOL/gm/hour	
			CONTROL	ACTH
38	A* + F+	1650	5.5	17.5
38	A	1956	8.0	19.0
	F	2400	4.0	6.3
36	A	1140	3.0	6.2
	F	1642	3.3	3.3
30	A	710	3.0	13.0
	F	544	10.0	8.1
25	A	630	14.0	20.4
	F	320	14.0	14.0
22	A	500	7.1	10.2
	F	330	6.0	10.0
20	A + F	320	14.0	14.0

* A = Adult Cortex + F = Foetal Zone

tions of tissue of equal weight (856 mg) were obtained from these glands. To one of these portions ACTH was added; the second served as a control. The extracts of the incubation media of the 3 controls and of the 3 ACTH-stimulated portions were separately pooled and chromatographed in the B_5 system. A photoprint of this first chromatogram is presented in Figure 4. The cortisol fraction was eluted and successively rechromatographed in the E_2B and B_5 systems. The results showed that the amount of cortisol (measured as Porter-Silber chromogen) produced by the control was equal to 11.0 µg/100G./hr., whereas the amount produced in the presence of ACTH was equal to 36.0. The specific activities of these two fractions were equal to 1990 and 870 c.p.m./µg respectively.

The results indicate that as early as the 22nd week of gestation ACTH promotes a distinct increase of cortisol production.

In summary, incubations in the presence of ^{14}C progesterone of slices of adult cortex or foetal zone from the adrenals of newborn infants produced ^{14}C cortisol and ^{14}C corticosterone from the foetal age of 22 weeks to term. In addition both the foetal zone and the adult cortex produced trace amounts of steroids which have been tentatively identified as desoxycorticosterone, 17α-hydroxyprogesterone and 11-desoxycortisol. The pattern of steroids produced from ^{14}C progesterone was remarkably similar in both types of tissue over the range of gestational age studied (22 - 36 weeks) and a predominance of C-21 steroids was apparent.

The quantitative results obtained from the study of six pairs of adrenals from five premature and one full term newborn infant indicate that the production of cortisol and corticosterone by the adult cortex is greater than by the foetal zone. Incubation of individual glands without added progesterone failed to produce sufficient amounts of steroid to

permit characterization and measurement. By pooling extracts from several incubations it was possible to measure the amount of cortisol present. The results suggested that the production of this steroid by both the adult and the foetal zones was stimulated by ACTH.

III. PLASMA CORTISONE AND CORTISOL LEVELS AT BIRTH AND DURING THE NEONATAL PERIOD

Assessment of adrenocortical function during the neonatal period has, up to the present time, relied chiefly on measurement of 17-hydroxy-corticosteroids in the neutral lipid extract of plasma and urine (171), yet, at variance with observations made in adults (137,139) the values obtained for unconjugated 17-hydroxycorticosteroids in the plasma of newborn infants do not agree well with those obtained for cortisol (127,145,158). The reason for this discrepancy is not completely understood. It may reflect the persisting influence of metabolic processes of maternal, placental or foetal origin.

A meaningful approach to the study of adrenocortical function during the neonatal period seems to require the quantitative assessment of individual steroids. The present study involved the development of a method (174) which fulfills this need. This method has here been applied to the re-evaluation of such problems as the concentration of cortisol in maternal and cord plasma and the concentration of cortisol from birth to the third day of life. The responsiveness of newborn adrenal to ACTH stimulation has also been investigated.

Unexpectedly it was found that cortisone was present in relatively high concentration in maternal, cord and newborn plasma. This finding was soon to be substantiated by the preliminary report of Seely (127) and by the study of Bro-Rasmussen (82).

In the various aspects of the present investigation cortisol concentration was systematically studied in relation to cortisone concentration in the hope of obtaining more information regarding the possible origin and metabolic significance of the latter compound.

A. METHODS

1. Material.

Dichloromethane, reagent grade, was washed with one volume of distilled water, dried over anhydrous sodium sulfate, filtered, refluxed 60 minutes over potassium hydroxide, and redistilled.

The reagents used for acetylation, dilution of ^{3}H acetic anhydride; and oxidation were purified as described by Kliman and Peterson (175).

The reagents used for chromatography were redistilled once in an all glass apparatus fitted with a Vigreux column.

Whatman No. 2 chromatography paper obtained from W. and R. Balston Ltd., London, England, was used unwashed. Steroids were eluted from paper with redistilled ethanol and applied on the chromatograms with dichloromethane.

All reference steroid acetates used as "internal pilots" (see below) were obtained from commercial sources and their purity checked by chromatography in appropriate systems (165).

^{14}C model compounds: 4-^{14}C cortisol (specific activity 66.6 uc per mg) was purchased from the New England Nuclear Corp. Due to the unavailability of 4-^{14}C cortisone during the establishment of the method 4-^{14}C cortisol was oxidized to 4-^{14}C cortisone (175) (specific activity 66.6 uc per mg). In subsequent experiments 4-^{14}C cortisone (specific activity 12.5 uc per mg) was also purchased from the New England Nuclear Corp. These steroids were rechromatographed every three to four months.

^{3}H acetic anhydride: specific activity 400 mc/mmole (New England Nuclear Corp.) was diluted with 9 mmoles of non-radioactive acetic anhydride and brought to a final concentration of 12% in dry benzene. The reagent was stored in a desiccator at 5°C. Due to the presence of non-volatile contaminants which form during storage (172) the New England Nuclear Corp. has recently recommended that the reagent be redistilled at intervals of one to two weeks.

2. Determination of specific activity of Acetic ^{3}H Anhydride.

0.5 mg of non-radioactive cortisol was chromatographed for 9 hours in the system B_5 of Bush (164). Following elution, the steroid was concentrated at the tip of a glass-stoppered tube (acetylation tube, capacity: 7 ml), and placed in a vacuum desiccator for not less than 6 hours prior to acetylation. 25 µl of pyridine and 30 µl of acetic ^{3}H anhydride were added to the tube, which was tightly corked and incubated at 37° C. for 20 hours.

Following extraction (175) cortisol acetate was subjected to two successive chromatographies in systems I and II (Table V).

After elution from the second chromatogram, the specific activity of the steroid acetate was established on duplicate aliquots by counting of the radioactivity and by the Porter-Silber reaction (171). The bulk of the material was then oxidized with CrO_3 (175). The product of the reaction - cortisone ^{3}H acetate - was chromatographed in system III and its specific activity established. Under these conditions the specific activity of the cortisol ^{3}H acetate after two chromatographies should be identical to that of its oxidation product on a micromolar basis, and be of the order of 5.0×10^{7} d.pm/µmole.

3. Extraction and acetylation of plasma samples. (Figure 6)

All blood samples were collected in heparinized tubes. The blood was centrifuged immediately after collection and the plasma stored at -3°C. until the time of assay. Blood samples from normal newborn infants were obtained from superficial veins of the limbs or scalp. Maternal blood was taken from the antecubital vein, and mixed cord blood from the distal end of the severed umbilical cord.

1×10^{4} d.p.m. of 4-^{14}C cortisol and of 4-^{14}C cortisone in absolute ethanol were pipetted into a 35 ml ground-glass stoppered centrifuge

TABLE V

CHROMATOGRAPHY SYSTEMS*

System	SOLVENT COMPOSITION:	Reference
No. 1 (B-3)	Light petroleum (b.p. 80-100° C), benzene, methanol, water (666/333/800/200)	(164)
No. 2 (KP 3)	Cyclohexane, benzene, methanol, water (4/3/4/1)	(175)
No. 3 (E-4)	Isooctane, tert-butanol, methanol, water (500/225/225/50)	(176)
No. 4 (CCl_4)	Carbontetrachloride, methanol, water (100/100/25)	

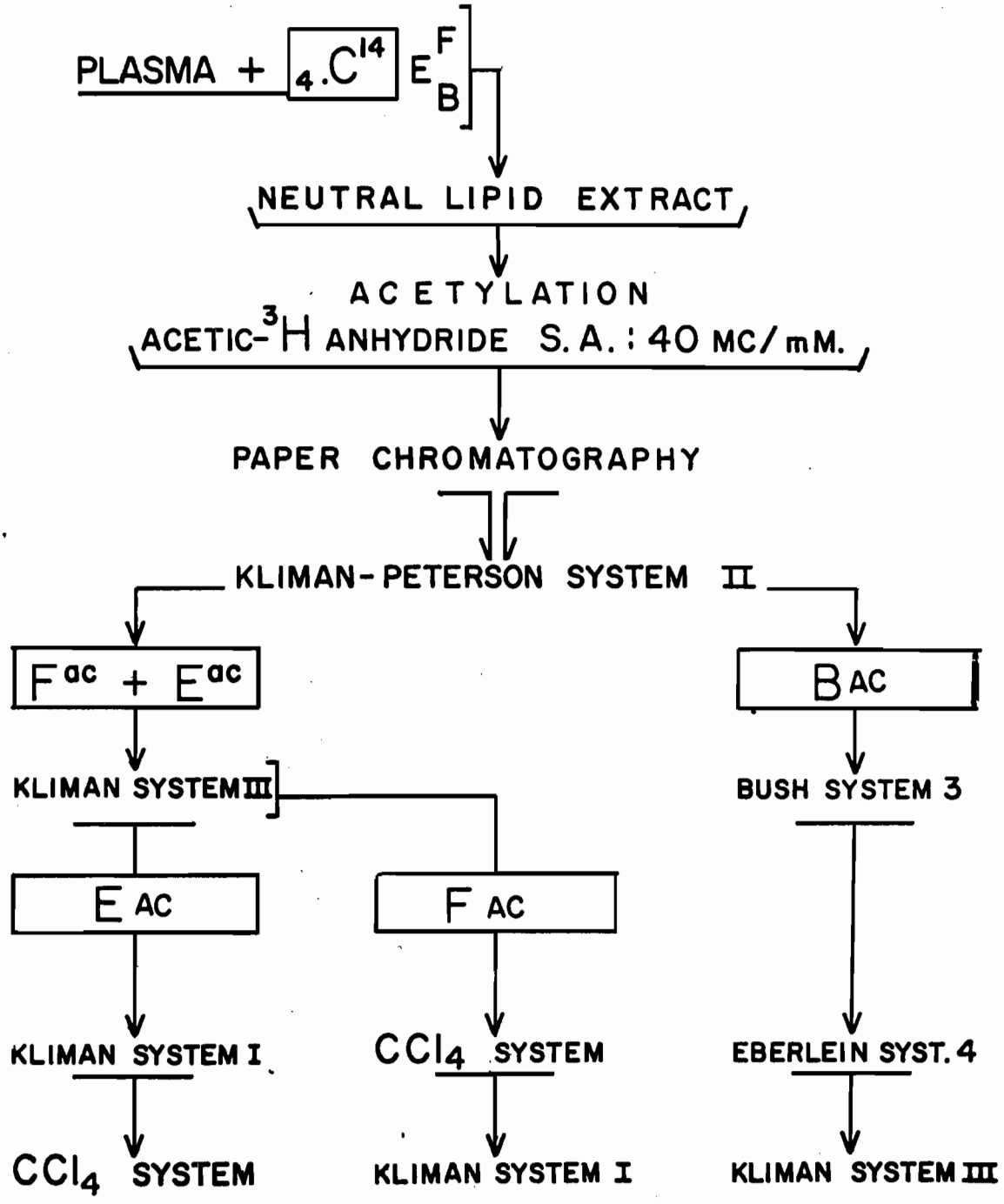
STEROID ACETATE MOBILITIES:

		System #1	System #2	System #3	System #4
Development:	(hr)	12	16	16	9
F acetate		5*	17	10	16
E acetate		10	28	10	24
B acetate		27	–	20	–

* The mobilities of the steroid acetates are expressed in cm. from the line of application to the center of the steroid spot visualized under ultra-violet light.

FIGURE 6

DOUBLE ISOTOPE DERIVATIVE ASSAY



tube and thoroughly dried. The plasma (average: 1.5 ml) was transferred to this tube and the steroids extracted into 10 volumes of ice-cold dichloromethane by vigorous shaking for 60 seconds. Emulsions, usually slight, were broken by centrifugation. The aqueous phase, together with a thin layer of coagulated protein, was removed by suction and the organic phase washed with 1/5 vol. of 0.1 N NaOH and then with water to neutrality (pH paper). The dichloromethane was filtered and evaporated to dryness under nitrogen. The residue was quantitatively transferred with ethanol and concentrated at the tip of an acetylation tube. Using an amount of plasma from 0.5 - 5 ml, this extraction procedure yields a small, clean residue. Following acetylation which was performed as described above, and extraction, 20 µg of the non-radioactive cortisol acetate and cortisone acetate were added to the dichloromethane extract. These "internal pilots" afford a simple means of detecting under ultraviolet light (253 mµ) the steroid areas to be eluted from subsequent chromatograms. Seven plasma samples and a saline blank were processed in each experiment. The use of a saline blank facilitated the calculations (see below), permitting a direct subtraction of the amount of ^{14}C steroids added to the plasma samples.

4. Paper chromatography.

All chromatograms were equilibrated and run at room temperature. The sequence of chromatography of the two steroid acetates studied, the composition of the chromatography systems, their times of development and the mobility of the steroids studied under these conditions are presented in Table V .

Following the separation of the acetates of cortisol, cortisone (and corticosterone) on the first system, the first two steroids were subjected to three further chromatographies. During the establishment of the method

the cortisol acetate and cortisone acetate fractions were oxidized with chromium trioxide (175) between the 3rd and 4th chromatograms; but since this procedure did not result in any change in the $^{3}H/^{14}C$ ratios it was omitted in subsequent studies. After elution from the 2nd, 3rd, and 4th chromatograms, an aliquot of each fraction corresponding to 1/5, 1/3, 4/5, was transferred to counting vials. The aliquots were thoroughly dried under infrared lamps prior to addition of 10 ml of scintillation fluid (4 g. of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(5 Phenyloxazolyl)-Benzene to 1 liter of toluene).

5. Tritium and carbon counting.

The measurement of radioactivity was performed in a Packard Automatic Tricarb Liquid Scintillation Spectrometer, Model 314-AX, operated at the single voltage of 1270 v. ^{3}H and ^{14}C counts were obtained and calculated according to a modification of the discriminator ratio method of Okita (173). Discriminator settings: upper scaler, 10 to 100 volts; lower scaler, 100 to infinity. Under these conditions, the contribution of the ^{14}C to the ^{3}H count was 50%, that of the ^{3}H to the ^{14}C count 3.5%.

The micrograms of free steroid per 100 ml of plasma were obtained from the following equation:

$$\mu g = \frac{^{3}H}{^{14}C} \times \frac{C}{SA} \times \frac{100}{V} \times \frac{MW}{1000} - n$$

where C = counts per minute of the 4-^{14}C cortisol or 4-^{14}C cortisone added.

SA = specific activity of the steroid acetate studied (c.p.m/mmole).

V = volume of plasma sample studied.

MW = molecular weight of the free steroid.

n = 4-^{14}C cortisol or 4-^{14}C cortisone added (μg).

The value of n may be obtained directly from the calculation of tritium associated with 4-^{14}C cortisol added to the saline blank or from the specific activity of the 4-^{14}C cortisol. On a molar basis the value of 4-^{14}C cortisol and of 4-^{14}C ^{3}H acetate should be equal, thus providing a further internal check on the method.

Constancy of isotope ratios (^{3}H/^{14}C) after the 3rd and 4th chromatographies was accepted as the criterion of identity of the unknown with the added ^{14}C tracer.

6. Critical assessment of the method.

The specificity and accuracy of the method were checked by studies such as that illustrated in Table VI. In this instance three 2 ml aliquots of a plasma sample obtained from a 3-day-old newborn infant were processed as follows: the first was used as control; to the second 0.45 ug of non-radioactive cortisol and 0.35 ug of cortisone were added; to the third twice these amounts of the same steroids. The recoveries of cortisol were 98 and 101%, those of cortisone: 102 and 104%.

From two pools of frozen plasma obtained from cord and maternal blood at the time of delivery, aliquots varying from 1-6 ml were taken over a period of 5 months for measurement of cortisol and cortisone. The results (Table VII) show that the reproducibility of the procedure is neither affected by the volume of the aliquot nor by prolonged storage at -5°C.

The sensitivity of the method, which is primarily dependent on the specific activity of the tritium acetic anhydride, and on the final recovery of the steroids, is also illustrated in Table VII. When 0.04 ug of cortisone was measured in 1 ml of plasma, the final

TABLE VI

RECOVERY OF CORTISOL AND CORTISONE ADDED TO PLASMA *

STEROID	PRESENT	ADDED	EXPECTED	OBTAINED	RECOVERY %
CORTISOL	0.18	0.45	0.63	0.63	101
CORTISOL	0.18	0.90	1.08	1.06	98
CORTISONE	0.12	0.35	0.47	0.49	104
CORTISONE	0.12	0.70	0.82	0.84	102

* In all instances values represent μg/sample.

TABLE VII

REPRODUCIBILITY AND SENSITIVITY OF THE METHOD

PLASMA SAMPLE	DATE OF ASSAY	PLASMA VOL. (ml)	CORTISOL $^{3}H/^{14}C$ Ratios After Chromatography 2nd	3rd	4th	µg/ALIQUOT	µg/%
MOTHER	AUG. 61	1	4.8	4.5	4.8	0.53	52.6
	JAN. 62	4	5.5	5.1	4.9	2.10	52.6
CORD	AUG. 61	1	1.8	1.6	1.6	0.20	19.8
	JAN. 62	3	3.1	3.1	2.9	0.64	21.3
INFANT (30 HRS)	AUG. 61	1	1.2	1.1	1.1	0.05	5.4
	JAN. 62	2	1.8	1.6	1.6	0.11	5.6
PLASMA SAMPLE	DATE OF ASSAY	PLASMA VOL. (ml)	CORTISONE $^{3}H/^{14}C$ Ratios After Chromatography 2nd	3rd	4th	µg/ALIQUOT	µg/%
MOTHER	AUG. 61	3	1.8	1.7	1.7	0.11	3.8
	JAN. 62	6	2.8	2.6	2.6	0.20	3.4
CORD	AUG. 61	3	3.3	2.6	2.6	0.20	6.8
	JAN. 62	5	2.4	2.7	2.7	0.34	6.7
INFANT (30 HRS)	AUG. 61	1	1.1	0.9	0.9	0.04*	4.2
	JAN. 62	2	1.5	1.4	1.4	0.08*	4.0

tritium count associated with this steroid was 261 counts per minute, representing 5 times the background count.

B. RESULTS

1. Cortisol and cortisone concentration in maternal and cord plasma.

Table VIII and Figure 7 present the concentration of cortisol and cortisone in maternal, and mixed umbilical cord plasma at the time of delivery, indicating the mean values, standard deviations and the ratio of cortisone to cortisol. In maternal plasma, the average cortisol concentration (52.4 ug%) was 6.5 times greater than in cord plasma (7.8 ug%). In contrast, in maternal plasma, the average cortisone concentration (5.2 ug%) was only one-third that of the cord plasma (13.6 ug%).

The difference between the concentration of cortisol and cortisone in the maternal and foetal-placental circulations is further illustrated by the ratios of cortisone:cortisol which in the maternal circulation averaged 0.1 and in cord plasma 1.8.

Analysis of this data, using the Pearson product-moment formula, indicates a significant correlation between the mother's cortisol and cortisone ($p < 0.05 \rightarrow 0.02$), and between the mother's cortisol and the cord cortisol ($p = 0.02$), and possible significance in the relation between the maternal cortisol and cord cortisone ($p < 0.1 \rightarrow 0.05$). The correlation coefficients do not suggest a significant relationship between the cord cortisol and cortisone or between the maternal cortisone and cord cortisone.

TABLE VIII

PLASMA CONCENTRATION OF CORTISOL (F) AND CORTISONE (E)
IN MATERNAL (M) AND CORD (C) PLASMA

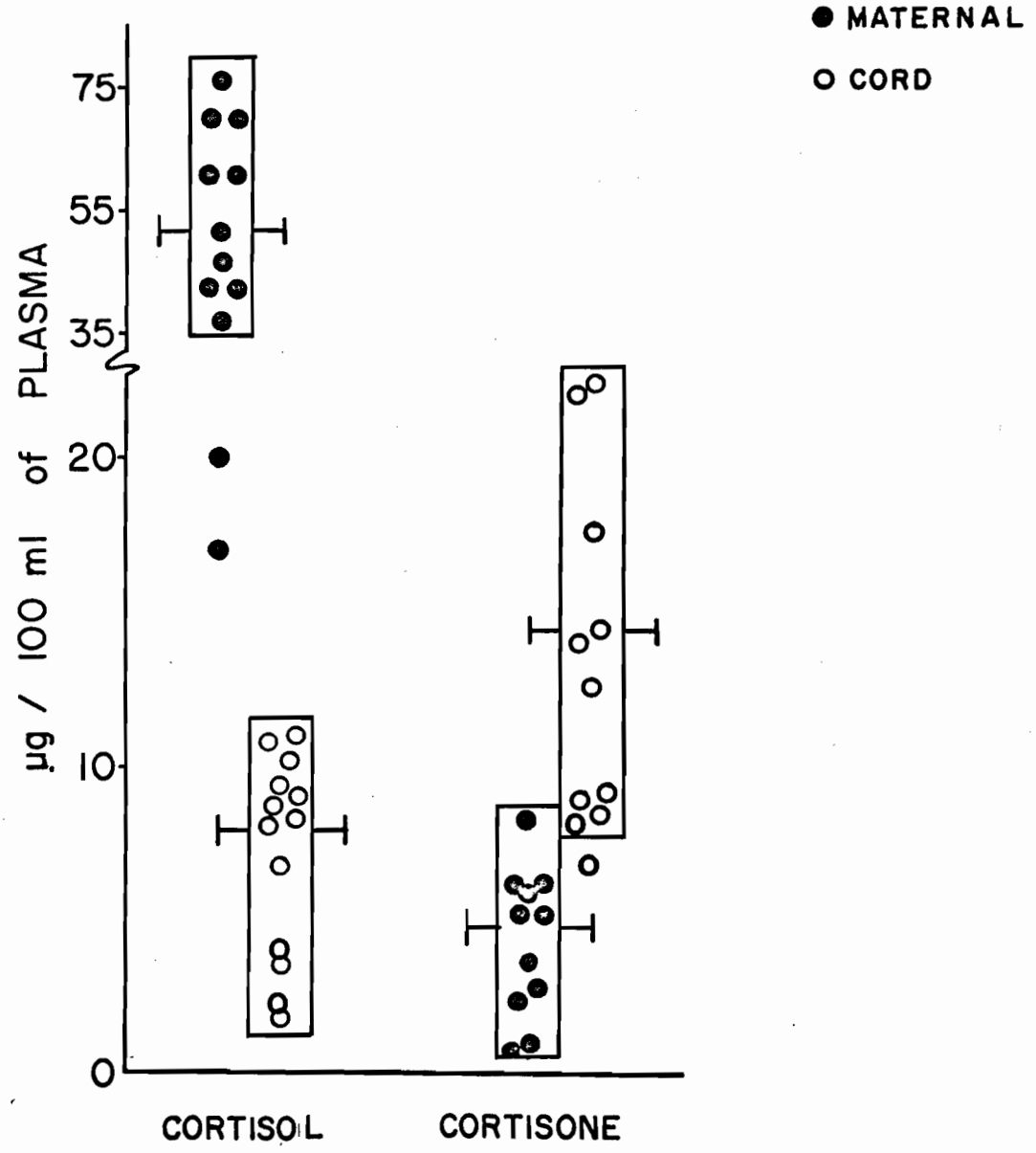
EXPER. NO.	CORTISOL μg/100 ml. Plasma		CORTISONE μg/100 ml. Plasma		RATIOS M	RATIOS C
	M	C	M	C	E/F	E/F
1	77.3	8.2	6.1	22.5	0.08	2.20
2	70.3	10.8	8.6	22.1	0.12	2.02
3	52.2	10.2	6.3	14.0	0.12	1.40
4	20.2	3.9	3.6	6.8	0.17	1.75
5	62.5	6.8	6.1	12.7	0.10	1.87
6	70.1	9.4	5.3	9.2	0.08	0.98
7	42.7	3.6	5.2	14.6	0.12	4.05
8	64.1	8.0	2.9	9.3	0.05	1.16
9	38.9	11.0	2.4	17.7	0.07	1.60
10	17.8	1.8	0.8*	8.5	0.05	4.70
11	40.1	1.7	7.8	8.8	0.20	5.18
12	74.5	18.9	7.6	16.3	0.10	0.86
MEAN	52.4	7.8	5.2	13.6	0.1	1.8
STANDARD DEVIATION	9.2	4.3	2.3	4.9	–	–

*For the measurement of plasma cortisone concentrations smaller than 1μg/100 ml., the plasma aliquot must be greater than 3ml. to fulfill acceptable criteria of accuracy.

FIGURE 7

CORTICOSTEROIDS IN MATERNAL AND CORD PLASMA

MATERNAL / CORD : 6·5 0·3



2. Plasma cortisol and cortisone concentration after birth.

Cortisol and cortisone concentrations measured in plasma samples obtained from the umbilical cord and from the infant at 6, 18, and 24 hours after birth are presented in Table IX and Figure 8. In spite of the limited number of observations it is apparent that the concentration of both steroids fell after birth. The values obtained at 18 hours of age are significantly lower than the concentration of cortisol ($p < .001$) and cortisone ($p < .005$) measured in cord plasma.

The concentration of cortisol and cortisone in random plasma samples obtained from newborn infants and from a small number of older infants is presented in Table X.

Although the number of measurements performed in plasma from infants after the first 48 hours is small, the values obtained suggest that cortisone remains a major blood corticosteroid for the first two weeks of life.

3. Cortisol and cortisone concentration following the administration of ACTH.

Table XI illustrates corticotropin responsiveness during the first three days of life. In each instance blood samples were obtained before, and six hours after, the intramuscular administration of 5 I.U. of ACTH (Duracton, Nordic Biochemicals).

A marked rise in plasma cortisol concentration was observed following ACTH in 9 of the 10 infants studied. The mean increase was 20.0 ug% ($p < 0.01$). A much smaller increase in the plasma cortisone concentration was observed with a mean difference between the control and ACTH values of 3.7 ug ($p < .001$). The higher 'p'

TABLE IX

CHANGES OF PLASMA CORTISONE (E) AND CORTISOL (F) IN NORMAL BABIES DURING THE FIRST 24 HOURS FOLLOWING DELIVERY*

PATIENT	CORD		6 HRS.		18 HRS.		24 HRS.	
	F	E	F	E	F	E	F	E
MARY	14.3	18.1	7.0	3.3	1.12	2.7	–	
SMITH	14.4	12.6	6.7	9.5	1.8	4.0	1.1	5.8
MICHEL	–	–	2.1	2.8	6.0	1.9	9.7	–
JOYCE	21.2	15.7	39.4	20.24	7.4	5.6	13.1	8.3
FRANCO	9.4	16.5	15.2	5.8	2.2	4.9	11.9	5.5
LIGNUS	16.8	8.4	7.2	3.4	4.8	3.9	–	–

* VALUES EXPRESSED AS MICROGRAMS OF STEROID PER 100 ML. PLASMA.

FIGURE 8

PLASMA CORTISONE AND CORTISOL IN FIVE NEWBORNS FROM BIRTH TO THIRTY HOURS OF AGE

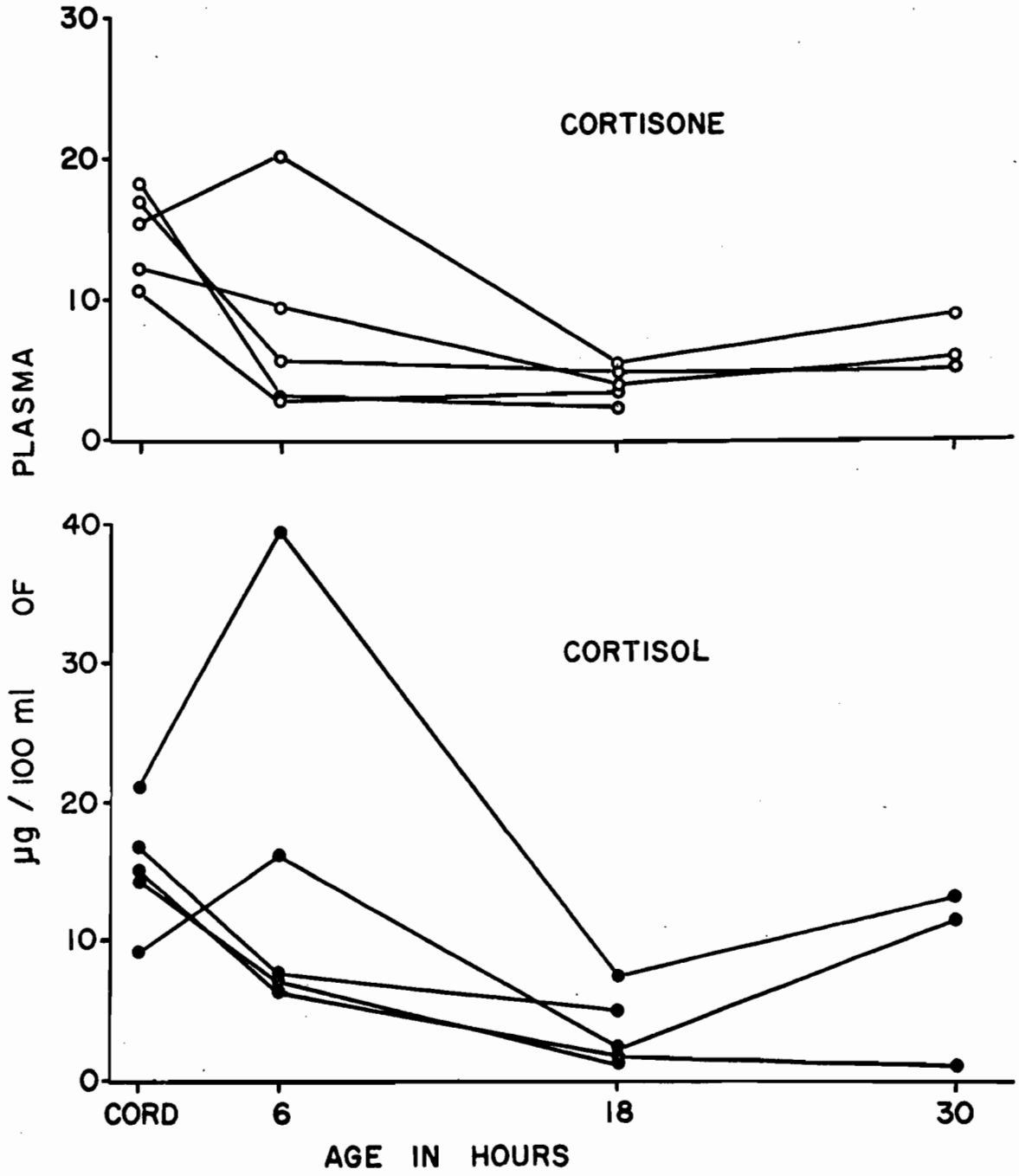


TABLE X

CORTISOL AND CORTISONE CONCENTRATIONS
IN RANDOM PLASMA SAMPLES

AGE	NO. OF PATIENTS	CORTISOL: μg %			CORTISONE: μg %		
		MEAN	RANGE	SD	MEAN	RANGE	SD
0-12 HR	9	7.1	3.3-19.8	3.15	5.3	2.4-9.7	2.24
12-24 HR	10	3.8	1.0-10.7	3.00	4.5	2.4-9.6	1.90
24-36 HR	9	8.5	2.1-16.8	4.90	6.0	3.2-9.6	2.30
36-48 HR	9	4.2	1.5- 6.6	2.35	3.9	2.0-6.0	1.35
48-72 HR	3	4.7	2.5- 7.8	2.20	3.9	3.1-5.5	1.20
11-16 DAY	3	2.8	2.5- 3.4	0.62	3.0	*0.2-6.1	2.40
1-5 MTH	3	4.7	3.8- 6.2	1.04	0.6	* 0-0.9	0.40

* SEE FOOTNOTE TO TABLE VIII

TABLE XI

ACTH RESPONSE IN THE NEWBORN PERIOD

AGE (HRS)	CORTISOL : µg %			CORTISONE : µg %		
	CONTROL	ACTH	INCREASE	CONTROL	ACTH	INCREASE
16	2.8	21.0	18.2	2.9	7.9	5.0
18	1.7	9.9	8.2	3.3	7.8	4.5
24	11.7	27.2	16.5	7.3	7.9	0.6
24	1.0	19.1	18.1	3.6	8.8	5.2
36	16.8	81.7	64.9	3.4	8.2	4.8
40	6.3	17.9	11.6	3.8	8.4	4.6
42	4.3	11.6	7.3	6.0	8.3	2.3
57	2.5	2.4	-0.1	3.2	5.3	2.1
51	7.8	13.3	5.5	5.5	6.5	1.0
74	3.3	54.6	50.8	3.1	9.6	6.4
MEAN VALUES	5.9	25.9	20.0	4.2	7.9	3.7

value obtained from the analysis of the cortisol response was due to the very wide range of values observed. (Figure 9)

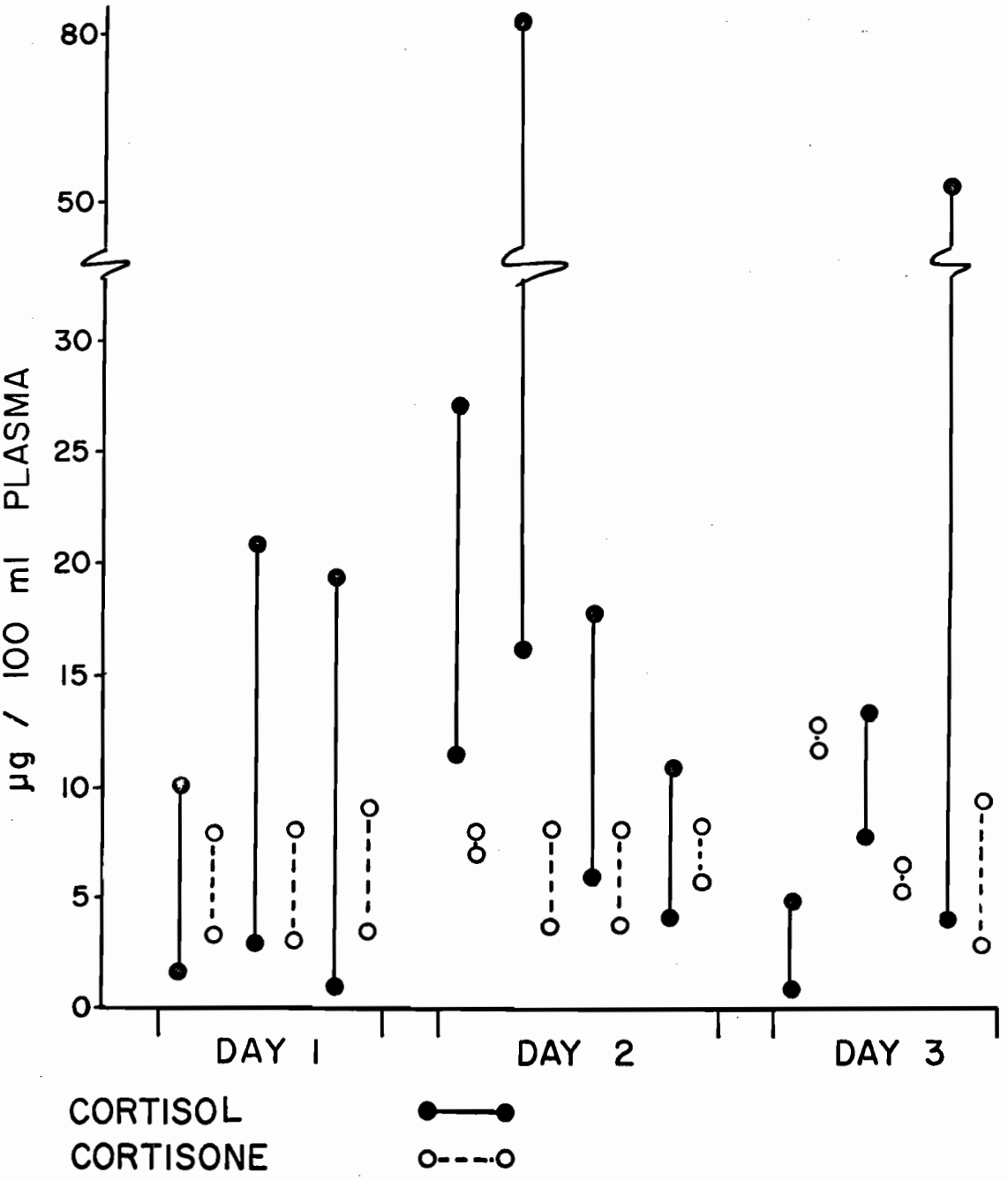
In summary, the study reports the measurement of cortisol and cortisone in maternal and cord plasma, as well as in newborn plasma, by a double isotope dilution derivative assay. An assessment of the reproducibility, precision and sensitivity of the method is presented.

It was found that cortisone concentration in cord plasma is greater than in maternal plasma, that following birth the plasma level of cortisone is maintained during at least the first week of life, and that both cortisol and cortisone concentration increased following the administration of ACTH.

FIGURE 9

ACTH TESTS

DURING THE FIRST 3 DAYS OF LIFE



In the course of the preceding study we observed that cortisone was present in mixed cord plasma, in maternal plasma and in the plasma of newborn infants during the first days of life. The 11β-hydroxy reduction of maternal cortisol during the transfer of this steroid across the placenta appeared to be a possible explanation for the presence of cortisone at least in the cord plasma. Sylbuski (113) reported a consistent yield of cortisone on incubation of cortisol with placental mince and evidence for the presence of 11 -hydroxyde-hydrogenase activity in placental subcellular fractions was presented by Meigs (114) and Osinski (115).

The present investigation was undertaken to study further the kinetics of this dehydrogenase in the microsomal fraction of placental homogenate.

A. METHODS

Placenta was obtained at the time of delivery and immediately placed in a plastic bag filled with saline and packed in ice. Upon arrival at the laboratory the placenta was freed from foetal membranes and repeatedly washed in 0.9% saline. The tissue was cut in roughly cubical pieces of 1.0 to 1.5 cm. The foetal membranes remaining attached to the parenchyma were excluded. The tissue was then shredded with forceps and the larger blood vessels removed. The resulting mince was rinsed several times with saline, blotted on filter paper, weighed and homogenized. The whole procedure was performed in a cold room at a temperature ranging between 4^{o} and $8^{o}C$.

To each gram of placental mince 9 ml of 0.25 M sucrose was added and the mixture was homogenized twice for 60 seconds in a Virtis homogenizer operated at top speed. After the first 60 seconds homogenization was interrupted 5 minutes to allow re-chilling of the suspension. Subcellular fractions were obtained by differential centrifugation at 2°C. in a Serval centrifuge (Model RC-2, Rotor: 55-34, in which r = 4.25 inches), according to the method of Hodgeboom (180), adopted with little modification. The nuclei, mitochondrial and microsomal fractions were obtained at 700 x g, 5000 x g, and 48,000 x g respectively. The time of centrifugation for the sedimentation of microsomes was 4 hours. On the basis of protein nitrogen the pellet of microsomes obtained by this prolonged centrifugation was quantitatively identical to that obtained by centrifuging 1 hour in the Spinco ultracentrifuge (Model L, Type 40 Rotor), operated at 105,000 x g.

In most instances only part of the homogenate (equivalent to 2 - 1 g. of placenta wet weight) was subjected to differential centrifugation, the bulk being frozen for further studies. Freezing of this homogenate for up to six weeks did not cause significant loss of 11 -hydroxydehydrogenase activity.

The tritium steroid substrates used (cortisol, cortisone and corticosterone 1,2,-^{3}H) were purchased from commercial sources (New England Nuclear Corp. or Tracerlab Inc.), or obtained from these companies as gifts of the Endocrine Study Section of the National Institutes of Health. All radioactive steroids were rechromatographed before use in appropriate systems (164,176).

Prior to the addition of the incubation mixture, the steroid in ethanol was pipetted into the incubation flask and the ethanol dried under air. Aliquots of the placenta subcellular fractions, usually equivalent to 200 - 500 mg of wet weight, were then added and diluted to a constant volume of 5 ml with 0.05 M phosphate buffer, pH 7.4, with or without cofactors. After flushing with O_2-5% CO_2 for 10 seconds, the vials were sealed and the incubation performed in a Dubnoff Metabolic Shaker Incubator for 2 hours at 37°C.

11β-hydroxydehydrogenase activity was assessed by double isotope assay of the 11-ketosteroid formed from the corresponding 11β-hydroxylated substrate. Following incubation, tracer amounts (about 6000 d.p.m.) of 4-^{14}C cortisol were added to the incubation mixture, which was then extracted with 7 volumes of redistilled dichloromethane. The organic phase was washed with 0.1 vol. of N NaOH and with H_2O to neutrality. After addition of internal steroid standards (15 µg each of cortisol and cortisone) the extract was first subjected to paper chromatography in the system B_5 of Bush (165) (development time: 4 hours). The steroids were visualized by scanning under an ultraviolet light (253 mµ) and eluted by soaking 10 minutes in ethanol. The fractions were then applied on the system E_2B of Eberlein (development time: 12 hours)(176). Following elution from this second chromatogram the steroids were acetylated overnight with 0.15 ml of acetic anhydride in 0.3 ml of pyridine. After evaporation of the acetylation mixture under air, the steroid acetates were subjected to a last chromatography in the system cyclohexane, benzene, methanol, water (4:2:4:1) of Kliman (175) (development time: 16 hours).

After each chromatography, aliquots of 1/10, 2/10, and 3/10 were taken for counting in a Packard Tri-carb liquid scintillation spectrometer, model 314-X. Each vial was routinely counted for 30 minutes at 1270 volts. Corrected ^{3}H and ^{14}C counts were calculated according to the method of Okita (173). Under such conditions the efficiency for ^{3}H and ^{14}C counting is 20 and 50% respectively. Constancy of the isotope ratio ($^{3}H/^{14}C$) following the 2nd and 3rd chromatographies was interpreted as evidence of identity of the unknown with the added ^{14}C carrier (see Table XII). The ^{14}C count provided the means of correcting for experimental losses.

When steroids other than cortisol were used for the purpose of studying substrate specificity, slight modification of the above procedure had to be introduced.

B. RESULTS

1. Localization of 11β-hydroxydehydrogenase activity.

The conversion of cortisol to cortisone observed with whole homogenates and subcellular fractions of placenta is presented in Table XIII.

The results of this study indicated that on subcellular fractionation the activity of the whole homogenate resided chiefly in the microsomal fraction. The activity of the homogenate was destroyed by boiling and greatly increased by the addition of 2 μmole of TPN in all fractions containing enzyme activity.

Practically no conversion of cortisol to cortisone was observed in those incubations which contained only isolated nuclei, mitochondria or the soluble fraction. The reaction did not occur

TABLE XII

$^{3}H/^{14}C$ RATIOS OF STEROID EXTRACTED FROM THE INCUBATION MEDIA OF PLACENTAL MICROSOMES

	$^{3}H/^{14}C$ RATIOS AFTER CHROMATOGRAPHY		
	1st	2nd	3rd
CORTISOL	2.64 2.83	2.53 2.89	2.52 2.90
CORTISONE	3.87 3.50	3.86 3.42	3.88 3.40
CORTICOSTERONE	5.18 5.29	5.27 5.46	5.18 5.40
11-DEHYDRO-CORTICOSTERONE	2.22 1.21	1.97 1.16	2.02 1.20

TABLE XIII

LOCALIZATION OF 11 β-HYDROXYDEHYDROGENASE ACTIVITY IN SUBCELLULAR FRACTIONS OF PLACENTA: EACH FRACTION EQUIVALENT TO 500 mg. OF TISSUE

CELL FRACTION	TPN μ MOLE	TOTAL RADIOACTIVITY RECOVERED %	SUBSTRATE CORTISOL	PRODUCT CORTISONE
			% OF TOTAL RADIOACTIVITY RECOVERED	
HOMOGENATE	0	81.8	79.1	20.9
	2	63.3	17.2	82.8
BOILED HOMOGENATE	2	76.4	98.4	1.6
NUCLEI	0	89.3	99.6	0.4
	2	71.1	100.0	0.0
NUCLEI-FREE HOMOGENATE	0	84.2	81.6	18.4
	2	72.4	42.4	57.6
MITOCHONDRIA	0	88.7	100.0	0
	2	72.6	100.0	0
SUPERNATE OF MITOCHONDRIA	0	77.3	84.7	15.3
	2	78.3	42.2	57.8
MICROSOMES	0	87.1	91.3	8.7
	2	81.6	65.9	44.1
SOLUBLE	0	82.4	98.7	1.3
	2	81.1	98.2	1.8
MICROSOMES PLUS SOLUBLE COMBINED	0	99.4	80.8	19.2
	2	68.4	40.5	59.5
MITOCHONDRIA PLUS SOLUBLE RECOMBINED	0	69.6	100.0	0
	2	66.8	100.0	0

Placenta preparations equivalent to 500 mg (wet weight) of tissue per flask. Final volume of the incubation mixture was brought to 5 ml. by adding 0.05 M phosphate buffer pH 7.4 and 0.04 M nicotinamide. Substrate : 2.0 μg of 1,2 ^{3}H Cortisol - SA 14.5×10^{3} c.p.m. /μg (Scintillation counter efficiency for ^{3}H : 20%). The mixture previously flushed for 10 seconds with O_2 - 5% CO_2 was incubated for two hours in a sealed flask. Extraction and assays of product and unaltered substrate were carried out by double isotope assay as described in the text. The figure of total radioactivity recovered (column 3) represents the count associated with cortisol and cortisone (corrected for loss during extraction and chromatography) as per cent of the total count of substrate cortisol added prior to incubation. The nuclei, mitochondria and microsome fractions were obtained at 700, 5000, and 48,000 xg. the latter after 4 hours of centrifugation.

in incubations performed with plasma or red blood cells obtained from mixed cord blood (4 ml in each case).

In the presence of TPN the conversion observed when the microsomal fraction was recombined with the soluble fraction was equivalent to that of the nuclei-free homogenate and to that of the supernatant of mitochondria, but slightly greater than that of the microsomal fraction alone.

2. Enzyme kinetics.

The conversion of cortisol to cortisone by placental microsomes in relation to microsomal protein concentration, time of incubation, changes of pH and addition of either TPN or DPN are illustrated in Tables XIV to XVII and Figure 10.

a) Effect of increasing microsomal protein concentration.

Increasing the microsome concentration in the incubation mixture from 3.99 to 19.75 mg of protein nitrogen produced a linear increase in the conversion of cortisol to cortisone (Table XIV). Above this concentration no further increase in conversion occurred. 90% of the activity of these preparations was destroyed by leaving the incubation mixture in a boiling water bath for 5 minutes.

b) Time course of 11β-hydroxydehydrogenation.

The time course of this enzymatic reaction was linear for the first two hours. Prolongation of the incubation for an additional two hours produced little increase in product formation. (Table XV)

TABLE XIV

FORMATION OF CORTISONE BY PLACENTAL MICROSOMES AS A FUNCTION OF MICROSOMAL PROTEIN CONCENTRATION

MICROSOMES		TOTAL RADIOACTIVITY RECOVERED	SUBSTRATE CORTISOL	PRODUCT CORTISONE
PROTEIN NITROGEN mg.	PLACENTA EQUIVALENT (wet wt. mg)	%	% of total radioactivity recovered	
3.95	666	92.7	90.8	9.2
7.9	1332	98.9	84.1	15.9
11.85	1998	95.1	78.6	21.4
19.75	3332	90.1	69.9	30.1
31.60	5332	94.4	67.9	32.1
11.85 (boiled)	1998	95.0	97.8	2.2

Placental microsomes in the amounts indicated were incubated for two hours with 12.0 µg of 1,2^{3}H cortisol (SA = 10.5 c.p.m./µg) 2 µmole of TPN and 0.04 M nicotinamide. For further details see legend of Table XIII.

TABLE XV

EFFECT OF TIME ON THE CONVERSION OF CORTISOL TO CORTISONE BY THE 11 β-HYDROXYDEHYDROGENASE OF PLACENTAL MICROSOMES

DURATION OF INCUBATION (min.)	TOTAL RADIOACTIVITY RECOVERED %	SUBSTRATE CORTISOL	PRODUCT CORTISONE
		% OF TOTAL RADIOACTIVITY RECOVERED	
10	89.6	98.7	1.3
30	91.7	97.1	2.9
45	93.7	94.8	5.2
60	89.7	93.0	7.0
90	91.7	90.7	9.3
135	71.3	86.9	13.1
180	89.0	85.4	14.6
240	78.6	84.8	15.2
135 (boiled)	88.0	98.6	1.4

Microsome preparations equivalent to 200 mg of placenta and to 4.75 mg of protein nitrogen in 0.05 M phosphate buffer, pH 7.4 and nicotinamide were incubated for the times indicated with 5 µg of 1,2-^{3}H cortisol (SA= 7.51 x 10^3 c.p.m./µg).
For further details see Table XIII.

c) Effect of pH.

In a study of the conversion of cortisol to cortisone as a function of changing pH the enzyme activity showed a broad maximum between pH 7.52 and 9.85. An abrupt fall in the total radioactivity recovered in the cortisol plus cortisone fractions was observed at pH greater than 8.06. This might reflect degradation of either the substrate or the product, or the formation of other products which remain unidentified. (Table XVI)

d) Effect of cofactors DPN and TPN.

The enzyme activity was increased by the presence of either TPN or DPN. As the concentration of either cofactor was raised from 0.05 to 0.5 μmole, a rapid increase in the conversion of substrate cortisol to product cortisone was observed, which tended to plateau at higher concentrations. As a cofactor TPN was twice as effective as DPN (Table XVII and Figure 10).

e) Substrate specificity.

The effect of increasing the concentration of the substrate cortisol alone and in the presence of a constant amount of corticosterone is shown in Table XVIII. The addition of 5 μg of corticosterone to the incubation mixture caused a reduction of the conversion of cortisol to cortisone throughout the range of substrate concentration tested.

To determine whether corticosterone inhibited the 11β-hydroxydehydrogenation of cortisol in a competitive or non-competitive manner the data was plotted according to Lineweaver and Burke (178).

TABLE XVI

EFFECT OF CHANGING pH ON 11 β-HYDROXYDEHYDROGENASE ACTIVITY OF PLACENTAL MICROSOMES

pH	TOTAL RADIOACTIVITY RECOVERED %	SUBSTRATE CORTISOL	PRODUCT CORTISONE
		% OF TOTAL RADIOACTIVITY RECOVERED	
5.15	88.2	92.7	7.3
6.93	85.5	83.1	16.9
7.52	83.5	76.4	23.6
8.06	80.9	75.1	24.9
8.55	63.9	72.0	28.0
8.98	40.6	70.0	30.0
9.85	46.1	73.2	26.8

Microsome preparations equivalent to 200 mg (wet weight) of placenta and to 4.75 mg of protein nitrogen in Tris buffer over the pH range indicated were incubated with 2µg of 1,2-^{3}H cortisol (SA= 14.5×10^{5} c.p.m./µg) and 2 µmole of TPN. For further details see Table XIII.

TABLE XVII

EFFECT OF INCREASING TPN AND DPN CONCENTRATION ON THE CONVERSION OF CORTISOL TO CORTISONE BY 11 β-HYDROXYDEHYDROGENASE OF PLACENTAL MICROSOMES

TPN μMOLE	DPN μMOLE	TOTAL RADIOACTIVITY RECOVERED %	SUBSTRATE CORTISOL	PRODUCT CORTISONE
			% OF TOTAL RADIOACTIVITY RECOVERED	
0.05	–	82.2	95.2	4.8
0.10	–	81.8	94.3	5.7
0.20	–	80.8	89.9	10.1
0.40	–	80.1	88.8	11.2
0.80	–	84.4	87.7	12.3
1.60	–	86.4	85.7	14.3
2.40	–	81.8	85.1	14.9
–	0.05	89.4	97.6	2.4
–	0.10	80.9	97.1	2.9
–	0.20	88.1	95.1	4.9
–	0.40	81.8	94.2	5.8
–	0.80	88.8	93.9	6.1
–	1.60	98.3	92.2	7.8
–	2.40	86.6	89.5	10.5
0.20	0.20	82.8	92.1	7.9
2.00	2.00	85.2	84.7	15.3

Microsome preparations equivalent to 200 mg (wet weight) were incubated with 5 μg of 1,2^{3}H cortisol (SA= 16.8 x 10^{3} c.p.m./μg). Nicotinamide was not present in the incubation and TPN or DPN were added in the amounts indicated. For futher details see the legend of Table XIII.

FIGURE 10

EFFECT OF INCREASING TPN AND DPN CONCENTRATION ON THE CONVERSION OF CORTISOL TO CORTISONE BY 11 β-HYDROXYDEHYDROGENASE OF PLACENTAL MICROSOMES.

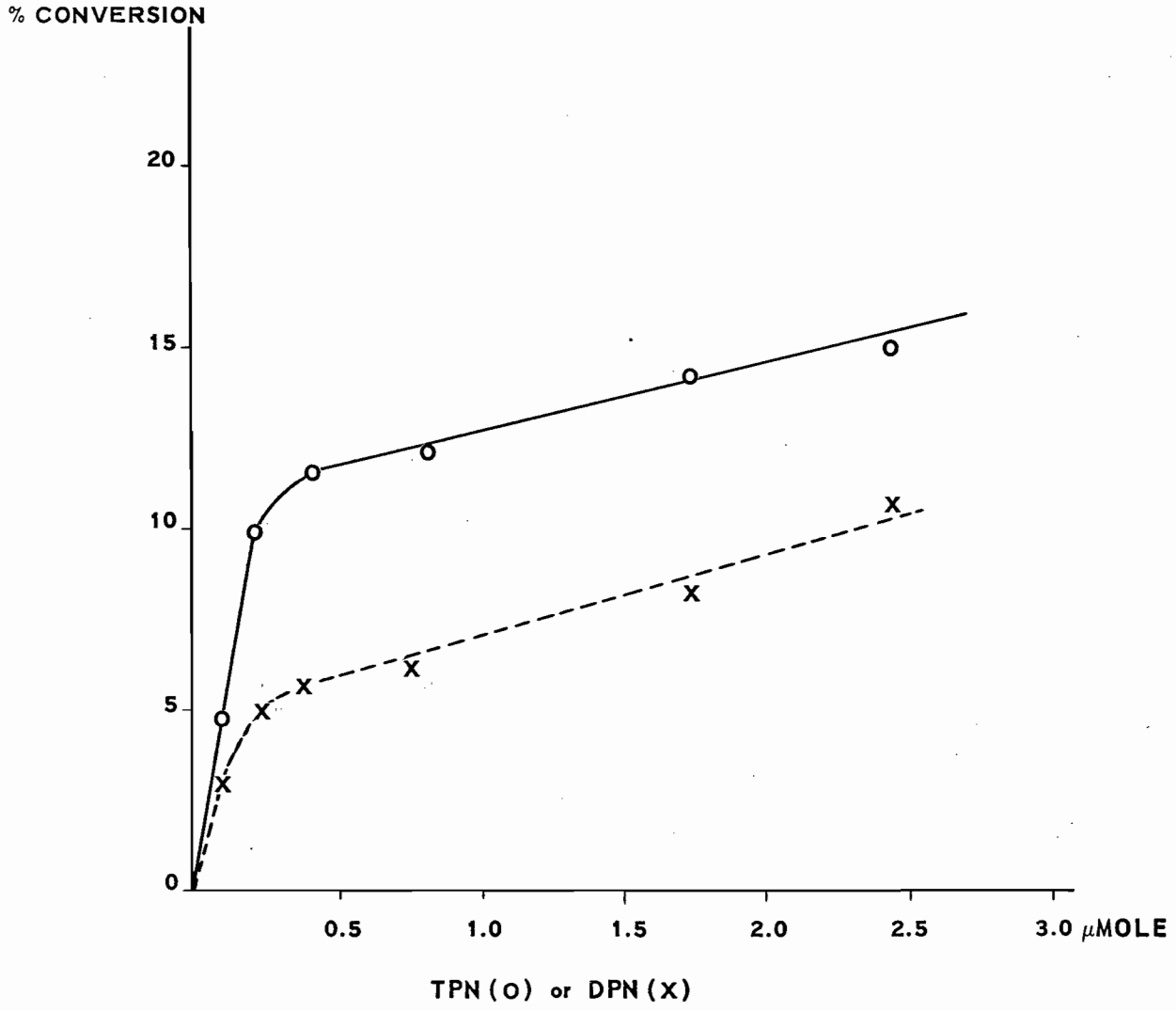


Figure 11 demonstrates that the inhibitor corticosterone increased the slope of the line without altering the intercept (1/V), indicating that the dehydrogenation of cortisol and corticosterone take place at the same reactive site.

In summary, an 11β-hydroxysteroid dehydrogenase was found associated with the microsomal fraction of human placenta obtained at term. The present work defines the activity of this enzyme preparation as a function of the duration of the incubation, the concentration of microsomes, the pH and the DPN or TPN concentration in the incubation media. Evidence was obtained that cortisol and corticosterone undergo 11β-hydroxydehydrogenation at the same active site of the enzyme.

TABLE XVIII

CONVERSION OF CORTISOL TO CORTISONE
AS A FUNCTION OF INCREASING SUBSTRATE
IN THE PRESENCE AND ABSENCE OF CORTICOSTERONE

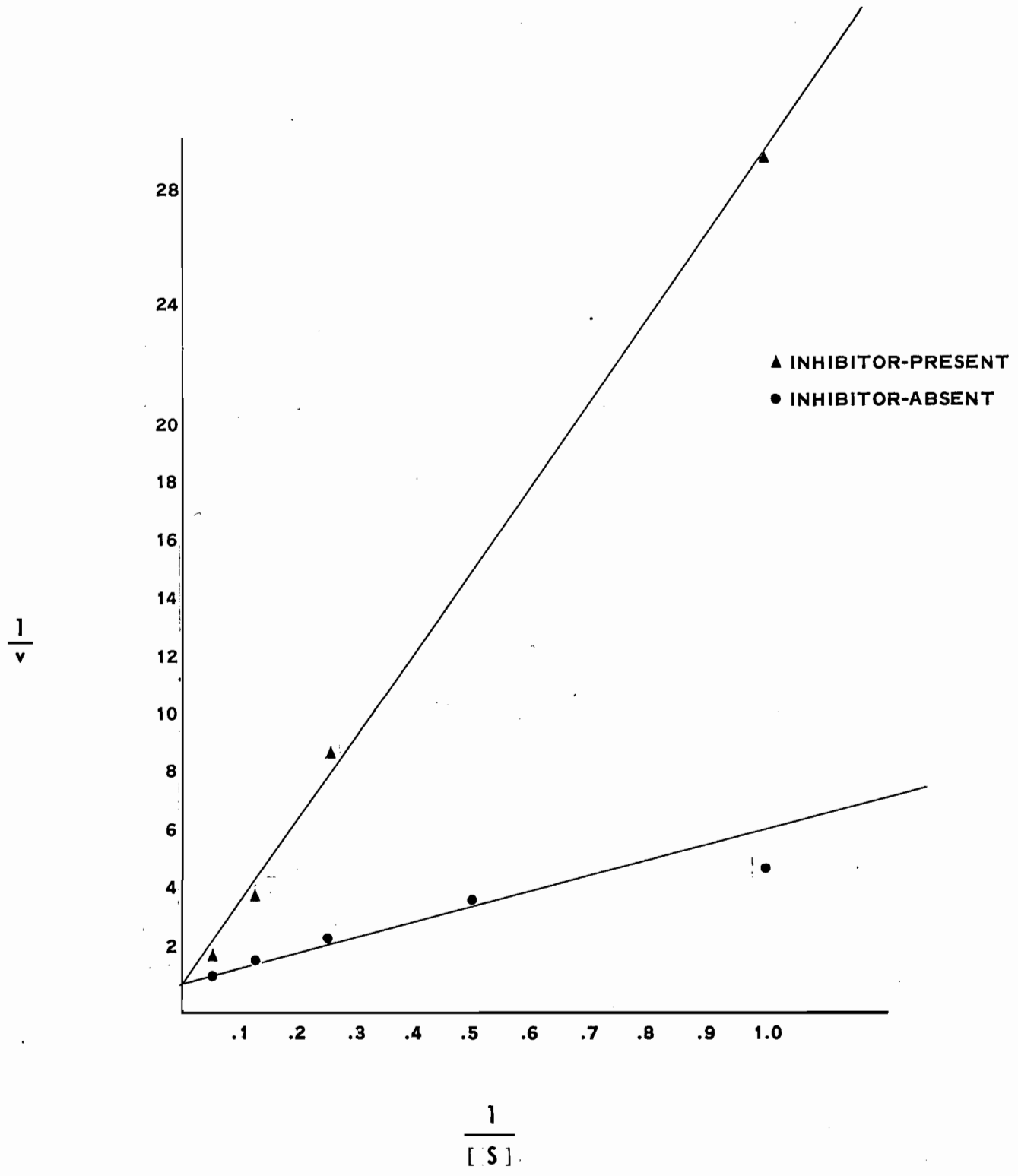
SUBSTRATE CORTISOL ug/flask	INHIBITOR CORTICOSTERONE ug/flask	PRODUCT CORTISONE ug/flask	LINEWEAVER BURKE CALCULATIONS	
			$\frac{1}{v^*}$	$\frac{1}{S^{**}}$
1	0	0.30	4.98	1.00
2	0	0.39	3.83	0.50
4	0	0.60	2.31	0.25
8	0	0.83	1.80	0.125
20	0	1.11	1.35	0.05
1	5	0.05	29.41	1.00
4	5	0.17	9.01	0.25
8	5	0.40	3.75	0.125
20	5	0.77	1.96	0.05

* v = Velocity of reaction cortisol cortisone expression as ug/hr.
** S = Substrate concentration ug/flask.

Microsome preparations equivalent to 200 mg (wet weight) of placenta and to 4.75 mg of protein nitrogen, in 0.05 M phosphate buffer containing 2 µmole of TPN were incubated with increasing concentrations of substrate cortisol with or without corticosterone over the ranges indicated.

FIGURE II

LINEWEAVER BURKE PLOT FOR THE 11 β-HYDROXYDEHYDROGENATION OF CORTISOL IN THE PRESENCE AND ABSENCE OF CORTICOSTERONE



D I S C U S S I O N

For a number of years the capacity of the foetal zone to produce cortisol and corticosterone had not been positively established nor were data available to indicate that cortisol production by this tissue was under ACTH control. In part this might have been due to the concept which attributed a predominantly androgenic function to the foetal zone. As a consequence, investigations were chiefly oriented towards the study of C-19 steroid biogenesis by this tissue. The formation of C-21 steroids by the foetal zone was suggested by experiments of Bloch (182) but technical difficulties prevented a definite conclusion concerning this point. Solomon (47), in studies involving the incubation of "mixed tissue" (adult cortex contaminated with foetal zone) in the presence of ^{14}C progesterone, observed seven bands of radioactive ultraviolet absorbing material which had separated on a chromatogram developed in the toluene-propylene glycol system. Of these, six were more polar than corticosterone. The authors did not identify these compounds nor did they indicate if they were produced in the incubation media of the foetal zone.

The results of the present investigation demonstrate that from the 22nd week of gestation to foetal maturity, both the adult and the foetal zones of the human newborn adrenal are capable of forming cortisol and corticosterone from progesterone. Although it is difficult to interpret these data in terms of endogenous steroid produc-

tion, in two experiments where no precursor was added, the analysis of pooled extracts of the incubation media of several groups of whole cortical slices or slices of either adult or foetal zone, revealed the chromatographic separation of measurable amounts of cortisol. The extremely small amounts of cortisol produced in the absence of added progesterone could be explained in several ways: the foetal adrenal may respond differently from the adrenal tissue of adults to the conditions of in vitro incubation, or the time of incubation may have been too short. Finally, this very low production could reflect a state of adrenal exhaustion preceding death. In this sense our results are comparable to those of Lanman (43) who reported only traces of formaldehydogenic or Zimmermann-reacting materials in the crude extracts of the incubation media of ten individual adrenals from premature infants.

It is difficult to decide whether our failure to demonstrate the production of C-19 steroids is related to the more advanced foetal age of the tissues studied, since Bloch (42,182) did not study the adrenals of foetuses beyond the 22nd week of gestation. However, it is more likely that the use of different precursors would have led more readily to the formation of these compounds. The studies of Solomon (179) indicate that adrenal androgens are mainly derived from 17α-hydroxypregnenolone by a biosynthetic pathway which does not involve the formation of progesterone.

The results of the present studies indicate the presence of 17, 11 and 21 hydroxylase activities in both the foetal zone and the adult cortex. It is thus conceivable that during foetal life these tissues are capable of producing corticosteroids from progesterone. If

insufficient amounts of progesterone were formed in the adrenal because of the "immaturity" of 3β-hydroxydehydrogenase activity, it is possible that placental progesterone could be utilized (1).

The studies also suggest that the production of cortisol by the foetal as well as the adult cortex is stimulated by ACTH. In several experiments the production of Δ^4-3 ketosteroids was increased by the addition of ACTH to the incubation media. Although the precision and specificity of this measurement are low, its validity in the in vitro bioassay of ACTH is well documented (166). Moreover, in two studies the action of ACTH on the production of cortisol by both the foetal zone and the adult zone was demonstrated following the separation of this steroid by paper chromatography.

These observations certainly imply the existence of 3β-hydroxydehydrogenase activity in the tissues studied, and are thus in keeping with the recent histochemical demonstration of this enzyme from the 8th week of gestation (184) and the observations of Villee (177) who demonstrated the formation of several corticosteroids of the Δ^4 pregnene series from pregnenolone. The report of Bloch (42) suggests also that ACTH stimulates the incorporation of sodium 1-^{14}C acetate into cortisol by foetal adrenals obtained as early as the 12th week of gestation. These data however are based on relatively few experiments.

The sensitivity and specificity of the double isotope derivative assay of different steroid hormones has been emphasized by previous investigators (126,175) and need not be discussed here. In

the studies of steroid metabolism in newborn infants, in which repeated blood sampling is often necessary, these methods are of particular value, permitting quantitative estimation of individual steroids in small aliquots of plasma. By means of such a method the present investigation confirms the higher concentration of cortisol in maternal than in cord plasma. This observation is in full agreement with the concept that the placenta acts as a barrier which allows only a fraction of the maternal cortisol to enter the foetal circulation. On the other hand, Bro-Rasmussen (126) has presented data comparable to ours on the concentrations of cortisone and on the difference of the cortisone/cortisol ratio in cord and in maternal plasma. Thus, a simple concentration gradient from the maternal circulation across the placenta cannot explain the higher concentration of cortisone in the foetal circulation.

The difference in cortisone concentration in the two circulations (foetal vs. maternal) could perhaps be explained if the foetal plasma proteins possessed a greater affinity to bind cortisone than maternal plasma proteins. This explanation seems to be unlikely, since preliminary studies, kindly performed for us by Dr. B. Murphy of the Queen Mary Veterans' Hospital (Montreal) (181) have indicated that the cortisone-binding capacity of maternal plasma is twice as great as that of cord plasma.

Statistical analysis of the cortisone and cortisol concentrations in each pair of maternal and cord plasma samples presented in Table VIII suggests that maternal cortisol may not only be transferred to the foetal circulation - as amply documented by Migeon (83,125) - but

may be metabolized to cortisone both by the mother and during transfer through the placenta. The existence of the latter metabolic process, namely an enzyme activity in the placenta which would account for the formation of cortisone, was previously studied by Osinski (115) and is here further defined. This 11β-hydroxysteroid dehydrogenase, localized in the microsomal fraction, is dependent on DPN or TPN as cofactors. In the presence of substrate cortisol and/or corticosterone, the formation of the product cortisone or 11-dehydrocorticosterone is related to the protein nitrogen content of the microsomal fraction, the concentration of substrate, the time of incubation and the concentration of cofactors. Based on Lineweaver-Burke calculations (178) the results demonstrate that cortisol and corticosterone, the two major Δ^4-unsaturated C-21 corticosteroids in adult human plasma, compete for the same active site of the enzyme. Considering the large size of the placenta at term, this organ could potentially dehydrogenate large amounts of cortisol and therefore account for the relatively high concentration of cortisone in cord blood. It is of interest to indicate here that an 11β-hydroxydehydrogenase system with the same cofactor requirement and subcellular localization has been described by Hurlock in rat liver (183), and that similar enzyme activity is also present in human liver microsomes (114).

One can only speculate on the physiological significance of cortisone in foetal plasma. In the maternal circulation, in spite of the increased transcortin, there is little doubt that the elevated level of non-bound cortisol is responsible for the mild signs of hypercorti-

cism noted in normal pregnancy (84). In foetal circulation the binding of cortisone being low, the level of free cortisone must be high, yet the newborn infant shows no clinical signs of adrenocortical hyperfunction. Bush has recently reviewed the evidence supporting the theory that the steroid-receptor interaction of glucocorticoids is dependent on a link between the 11β hydroxyl group and the receptor. The 11 ketonic group of cortisone appears to be inactive at the receptor site and becomes active only when reduced to cortisol. If one postulates that the foetus is unable to convert cortisone to cortisol one could explain not only why the foetus shows no signs of cortisol excess, but also understand how the 11β-hydroxydehydrogenase activity of the placenta could serve to protect the foetus from the high cortisol concentration in the maternal circulation.

This hypothesis does not however explain the persistence of appreciable concentrations of cortisone after birth and the definite increase in plasma cortisone levels following the administration of ACTH.

Although the possibility that cortisone is secreted by the newborn adrenal cannot be entirely ruled out, in the incubation of neonatal adrenal tissue reported above cortisone was not formed from ^{14}C progesterone.

The rapid conversion of cortisol to cortisone by the newborn, whether in the liver or possibly within the circulation, would appear to offer a more satisfactory hypothesis. Seely, in a preliminary communication, reported data similar to ours on the plasma concentration

of cortisone following birth (127,145) and demonstrated that, following the administration of 4-^{14}C cortisol to a newborn infant, the plasma concentration of 4-^{14}C cortisone exceeded that of 4-^{14}C cortisol 30 minutes after injection.

Since cortisone is not usually recognized as a normal component of plasma 17-hydroxycorticosteroids, one is entitled to speculate whether its presence in plasma during the first week of life is related to the ill-defined "immaturity" of enzymes which control the catabolic degradation of cortisol.

An impairment of Δ^4-reductase activity is suggested by the findings of Bongiovanni (19) who showed that in newborn infants the half-life of tetrahydrocortisol is normal, whereas that of cortisol is prolonged. If more direct evidence were obtained in favour of this "defect", it could in part explain the persistence of cortisone in infant plasma.

Finally, this study, together with the comprehensive investigations of Bertrand (122), leaves little doubt that the newborn infant responds normally to ACTH administration and can do so in the first hours of life.

CLAIM TO ORIGINAL RESEARCH

Of the studies presented in this thesis the following seem to fulfill best the criterion of originality:

1) The demonstration that C^{21} steroid biogenesis in the foetal zone is qualitatively similar to that in the adult cortex and that the production of cortisol by both zones is increased by ACTH.

2) The finding that cortisone is a major corticosteroid in foetal and neonatal plasma and that the concentration of this steroid in cord plasma is greater than in maternal plasma.

3) The presentation of kinetic studies of 11 B-hydroxy-dehydrogenase in human placenta which were not previously available.

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