METABOLISM OF DEHYDROEPIANDROSTERONE BY THE ADRENAL GLAND OF THE HUMAN FOETUS AND NEWBORN INFANT

by

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N O M E N C L A T U R E

ABBREVIATION	TRIVIAL NAME	SYSTEMATIC NAME
Δ^4A	androstenedione	4-androstene-3,17-dione.
11$\beta\Delta^4$A	11β-hydroxyandrostenedione	11β-hydroxy-4-androstene-3,17-dione.
A'diol	androstenediol	5-androstene-3β,17β-diol.
-	aldosterone	11β,21-dihydroxy-3,20-dioxo-4-pregnen-18-al.
-	androstanedione	5β-androstane-3,17-dione.
B	-	11β,21-dihydroxy-4-pregnene-3,20-dione.
BS	-	11β,21-dihydroxy-4-pregnene-3,20-dione 21-monosulphate.
-	cholesterol	5-cholesten-3β-ol.
20α-cholesterol	-	5-cholestene-3β,20α-diol.
17α,20α-cholesterol	-	5-cholestene-3β,17α,20α-triol.
11 -dehydroB	-	21-hydroxy-4-pregnene-3,11,20-trione.
DHA	dehydroepiandrosterone	3β-hydroxy-5-androsten-17-one.
DHAAc	DHA acetate	17-oxo-5-androsten-3β-yl acetate.

ABBREVIATION	TRIVIAL NAME	SYSTEMATIC NAME
DHAS	DHA sulphate	17-oxo-5-androsten-3β-yl sulphate.
7-DHA	7-hydroxyDHA	3β,7ξ-dihydroxy-5-androsten-17-one.
16∝DHA	16∝-hydroxyDHA	3β,16∝-dihydroxy-5-androsten-17-one.
16∝DHAS	16∝-hydroxyDHA sulphate	3β,16∝-dihydroxy-5-androsten-17-one 3-monosulphate.
DOC	-	21-hydroxy-4-pregnene-3,20-dione.
DOCS	-	3,20-dioxo-4-pregnen-21-yl mono-sulphate.
E_1	oestrone	3-hydroxy-1,3,5(10)-oestratrien-17-one.
E_2-17β	oestradiol	1,3,5(10)-oestratriene-3,17β-diol.
E_3	oestriol	1,3,5(10)-oestratriene-3,16∝,17β-triol.
E_3-16G	-	1,3,5(10)-oestratriene-3,16∝,17β-triol 16-β-D-glucopyranosiduronate.
E_3-3S,16G	-	1,3,5(10)-oestratriene-3,16∝,17β-triol 3-sulphate 16-β-D-glucopyranosiduronate
F	cortisol	11β,17∝,21-trihydroxy-4-pregnene-3, 20-dione.

ABBREVIATION	TRIVIAL NAME	SYSTEMATIC NAME
FS	-	11β,17α,21-trihydroxy-4-pregnene-3, 20-dione 21-monosulphate.
16αF	-	11β,16α,17α,21-tetrahydroxy-4-pregnene-3,20-dione.
-	(11-oxoandrostenedione)	4-androstene-3,11,17-trione.
3αP	-	3α-hydroxy-5β-pregnan-20-one.
3α,20αP	-	5β-pregnane-3α,20α-diol.
$3\alpha,20\alpha P^{S}$	-	5β-pregnane-3α,20α-diol 3 monosulphate.
$\Delta^{4}P$	progesterone	4-pregnene-3,20-dione.
$11\beta\Delta^{4}P$	11β-hydroxyprogesterone	11β-hydroxy-4-pregnene-3,20-dione.
$16\alpha\Delta^{4}P$	-	16α-hydroxy-4-pregnene-3,20-dione.
$17\alpha\Delta^{4}P$	17α-hydroxyprogesterone	17α-hydroxy-4-pregnene-3,20-dione.
$20\alpha\Delta^{4}P$	-	20α-hydroxy-4-pregnen-3-one.
$20\beta\Delta^{4}P$	-	20β-hydroxy-4-pregnen-3-one.
$11\beta,17\alpha\Delta^{4}P$	-	11β,17α-dihydroxy-4-pregnene-3,20-dione.

ABBREVIATION	TRIVIAL NAME	SYSTEMATIC NAME
17α,20αΔ^4P	-	17α,20α-dihydroxy-4-pregnen-3-one.
17α,20βΔ^4P	-	17α,20β-dihydroxy-4-pregnen-3-one.
Δ^5P	pregnenolone	3β-hydroxy-5-pregnen-20-one.
Δ^5PS	pregnenolone sulphate	20-oxo-5-pregnen-3β-yl sulphate.
16αΔ^5P	16α-hydroxypregnenolone	3β,16α-dihydroxy-5-pregnen-20-one.
17αΔ^5P	17α-hydroxypregnenolone	3β,17α-dihydroxy-5-pregnen-20-one.
17αΔ^5PS	17α-hydroxypregnenolone sulphate	3β,17α-dihydroxy-5-pregnen-20-one 3-monosulphate.
21Δ^5P	21-hydroxypregnenolone	3β,21-dihydroxy-5-pregnen-20-one.
16α,20αΔ^5P	-	5-pregnene-3β,16α,20α-triol.
17α,21Δ^5P	17α,21-dihydroxypregnenolone	3β,17α,21-trihydroxy-5-pregnen-20-one.
S	-	17α,21-dihydroxy-4-pregnene-3, 20-dione.
SS	-	17α,21 —dihydroxy-4-pregnene-3,20-dione 21-monosulphate
T	testosterone	17β-hydroxy-4-androsten-3-one.

ABBREVIATION	TRIVIAL NAME	SYSTEMATIC NAME
TAc	testosterone acetate	3-oxo-4-androsten-17β-yl acetate.
TS	testosterone sulphate	3-oxo-4-androsten-17β-yl sulphate.
6βT	6β-hydroxytestosterone	6β,17β-dihydroxy-4-androsten-3-one.
11βT	11β-hydroxytestosterone	11β,17β-dihydroxy-4-androsten-3-one.
11βTAc	11β-hydroxytestosterone acetate	11β,17β-dihydroxy-4-androsten-3-one. 17-monoacetate
16αT	-	16α,17β-dihydroxy-4-androsten-3-one

INTRODUCTION

The endocrinology of human pregnancy has been the subject of much study during the past century. Halban (1905) proposed that the placenta was the organ responsible for the hormonal changes which occur during gestation. This theory was generally accepted until recent years when attention was directed to the possibility of hormonal interactions between the placenta and the maternal and foetal endocrine glands (e.g. Davis & Plotz, 1956). Particular interest has been shown in the foetal adrenal glands which undergo striking morphological changes in the neonatal period. Perfusion studies of the foetus and placenta have yielded information which implicates the foetal adrenal glands as the principal source of precursors for the placental production of the large amounts of oestrogens which are excreted in the urine of pregnant women. In vitro incubations have demonstrated that the human foetal adrenal gland is capable of de novo steroid synthesis and of numerous steroid transformations. Indirect evidence that the foetal adrenal represents an important site of steroid biosynthesis and metabolism within the foeto-placental unit has been obtained by the identification and quantitation of steroids isolated from amniotic fluid and

the blood and urine of the pregnant woman, the foetus and the neonate. Little is known, however, about the sites of steroid formation within the foetal adrenal gland. The aim of the present study is the investigation of the steroidogenic capacities of the different histological zones of the adrenal gland of the human foetus and newborn infant.

The morphology of the human foetal adrenal gland.

The large size and post-natal involution of the human foetal adrenal gland has been known for several centuries (e.g. Winslow, 1766: "Elles sont chez le foetus extrêmement grosses et diminuent en volume avec l'âge."). Minst (1897) observed cells in the central portions of the glands which disappeared, taking no part in the formation of the adult organ. The existence in the adrenal gland of a histological zone which does not persist after the first few months of extra-uterine life was discovered by Starkel & Wegrzynowski (1910) and Thomas (1911). These authors reported that the adrenal cortex in the human foetus consists of two zones; a thin, outer "adult zone" and an inner "foetal zone", which occupies approximately 80% of the neonatal gland (Plate I). The adult zone differentiates after birth, the zona fasciculata being distinguishable from the zona glomerulosa after two to three weeks and

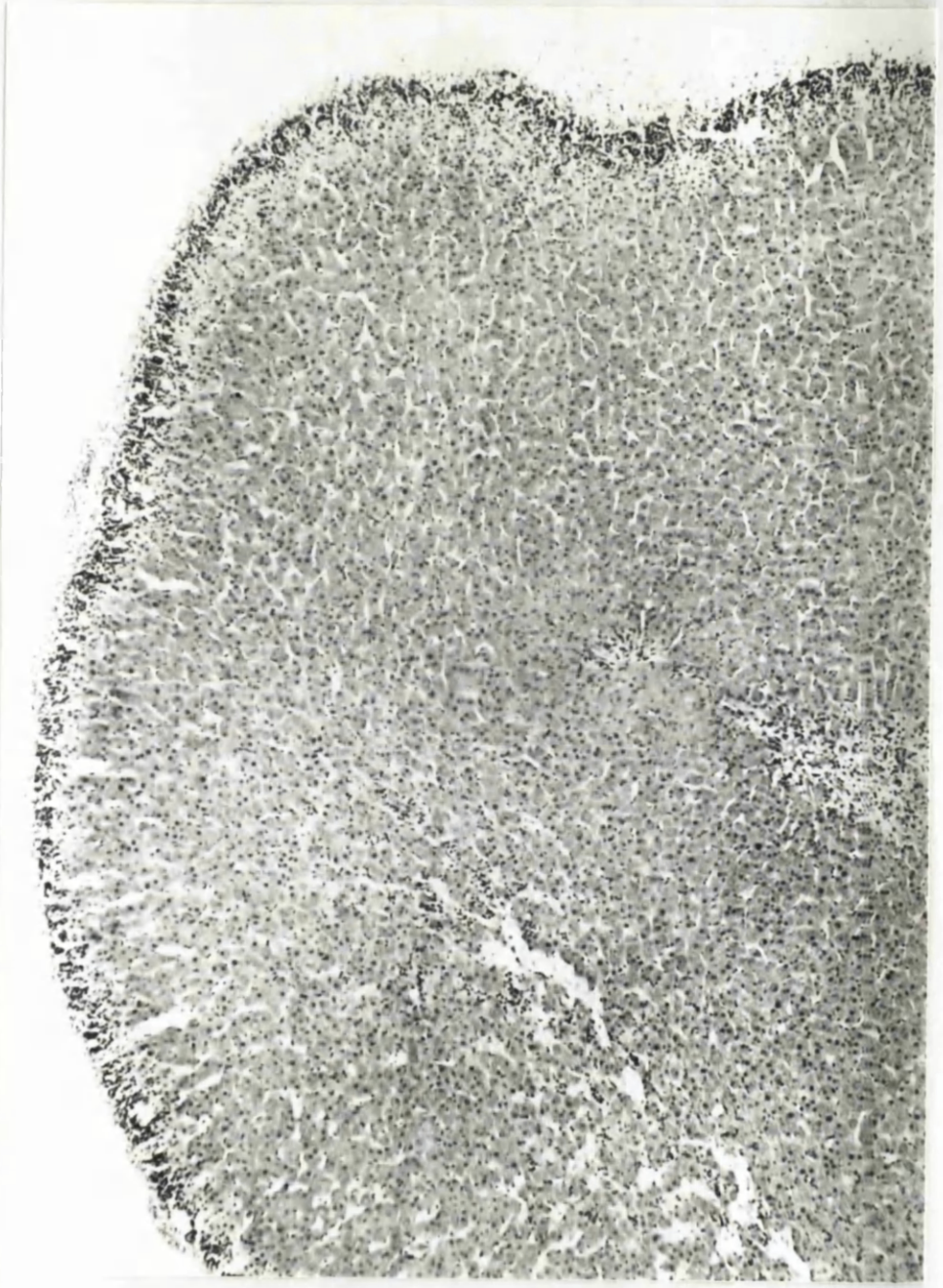


Plate I. Adrenal gland of a human foetus of 20 weeks gestation.
H. & E. (X 75).

the zona reticularis developing by three to three and a half months of life (Keene & Hewer, 1927; Bongiovanni, 1951). The foetal zone involutes post-natally and has disappeared by six to nine months of life (Tähka, 1951). Lewis & Pappenheimer (1916) reported degenerative changes in the foetal zone during the first few days after birth, followed by a massive degeneration after the second week of life, which resulted in the formation of a fibrous zone between the medulla and the developing adult zone.

The hypertrophy of the human adrenal glands was emphasized by Ekholm and Niemineva (1950) who calculated the adrenal weight:body weight ratio at birth as 1:394 compared with 1:7,000 in the adult. The maximum ratio (1:191) was attained during the fourth month of gestation when the foetal adrenal is as large as the kidney. Tähka (1951) observed scarcely any change in the weight of the adrenal glands during the first fourteen days of life, followed by a distinct drop in the fifteen to thirty day period, after which the weight fell continuously until two to three months of life. She confirmed the observations of Lewis & Pappenheimer (1916) that the development of the adrenal glands is similar in full-term and premature infants and displays no sex differences. Bongiovanni

(1951) also reported a reduction to half of the birth weight of the glands during the first two weeks of life, the neonatal weight not being regained until puberty.

The embryology of the human foetal adrenal glands.

There are conflicting reports in the literature concerning the origin of the zonal tissues of the foetal adrenal cortex. Keene & Hewer (1927) identified the anlage of the adrenal gland of 10 mm embryos as bilateral masses of cells situated medially to the mesonephric bodies and having a small free surface covered by coelomic epithelium. This, they suggested, formed a cap of cells three or four layers thick over the developing glands constituting the adult zone. Grollman (1936) designated the foetal zone, the "androgenic zone", and the adult zone, the "corticogenic zone", postulating that they produced androgens and corticosteroids respectively. This theory was supported by Vélican (1947, 1950) who proposed that the cells of the adult zone have the same embryological origin as the interstitial cells of the gonads, while the foetal zone cells derive from a separate region of the coelomic epithelium. Crowder (1957) reported that both zones share the same cellular components, the differences in

appearance being due solely to an increased rate of maturation of the foetal zone cells. The site of the anlage of the foetal adrenal gland proposed by Keene & Hewer (1927) was confirmed by Jirásek (1969), who observed a transformation of the cells in the centre of the adrenal blastema in 15 - 20 mm embryos to the epithelial cells of the foetal zone, the small blastematous of the outer layers forming the adult zone. The development of both zones from a common cell type has been proposed by other authors (Uotila, 1940; Dhom, Ross & Widok, 1958; Johannisson, 1968).

The ultrastructure of the human foetal adrenal gland.

Johannisson (1968) has performed an extensive study of the ultrastructure of the human foetal adrenal cortex. The adrenocortical cells of embryos of five to six weeks gestation are immature in appearance, developing at about six weeks into the adult and foetal zones. The cells of the latter zone, during the first trimester of pregnancy revealed an increase of endoplasmic reticulum, mitochondria and Golgi complex which she interpreted as indicative of a functional differentiation of this zone. No such indications of steroidogenic potential were apparent in

the cells of the adult zone until the last part of the second and the beginning of the third trimester. The depletion of osmiophilic material in the cells of the foetal zone observed during the last trimester was considered a result of increased steroid secretion. "Dark" cells appeared during the second trimester in the "transitional" area between the adult and foetal zones. These cells were of two types; one displaying a compact structure, and the other a dilatation, of the tubules of the smooth endoplasmic reticulum. Cells of varying electron opacity were previously noted in the human foetal adrenal glands by Ross (1962).

The adrenal gland in anencephaly.

Much interest has been created by the abnormality of the adrenal glands of anencephalic foetuses. Morgagni (1723) observed that if the cerebral hemispheres have failed to develop normally, the adrenal glands of the neonate are abnormally small. The adrenal weight:body weight ratio in newborn anencephalic infants is 1:1,535 compared with 1:307 - 1:417 in normal foetuses (Ekholm & Niemineva, 1950). Elliot & Armour (1911) found that the reduction in size results from atrophy of the foetal

zone, the glands being composed almost entirely of adult zone cells (see Plate II) and compared the appearance of the adrenal glands of the full-term anencephalic infant to a miniature of that observed in the one-year-old child. Histologically normal adrenal glands in anencephalic foetuses of two and four months gestation were described, however, by Meyer (1912). His observations are consistent with the report of Zander (1890) that the kidney volume: adrenal volume ratio was nearest normal in the youngest anencephalic foetuses which he studied. No evidence of premature involution of the foetal zone was obtained by McNeill (1947) who examined the adrenal glands of five anencephalic foetuses of varying maturity. Benirschke (1956), however, in a larger series of twenty-four such foetuses of nine to thirty-nine weeks gestation, observed that the foetal zone, after twenty weeks of gestation involutes in a comparable fashion to the normal neonatal gland, except that no fibrous septum develops between the adult zone and the medullary cells. The cells of the adrenal cortex of full-term anencephalic infants have little Golgi complex and mitochondria with an internal structure similar to that observed in the adult zone of normal foetuses of six to fourteen weeks gestation (Johannisson,

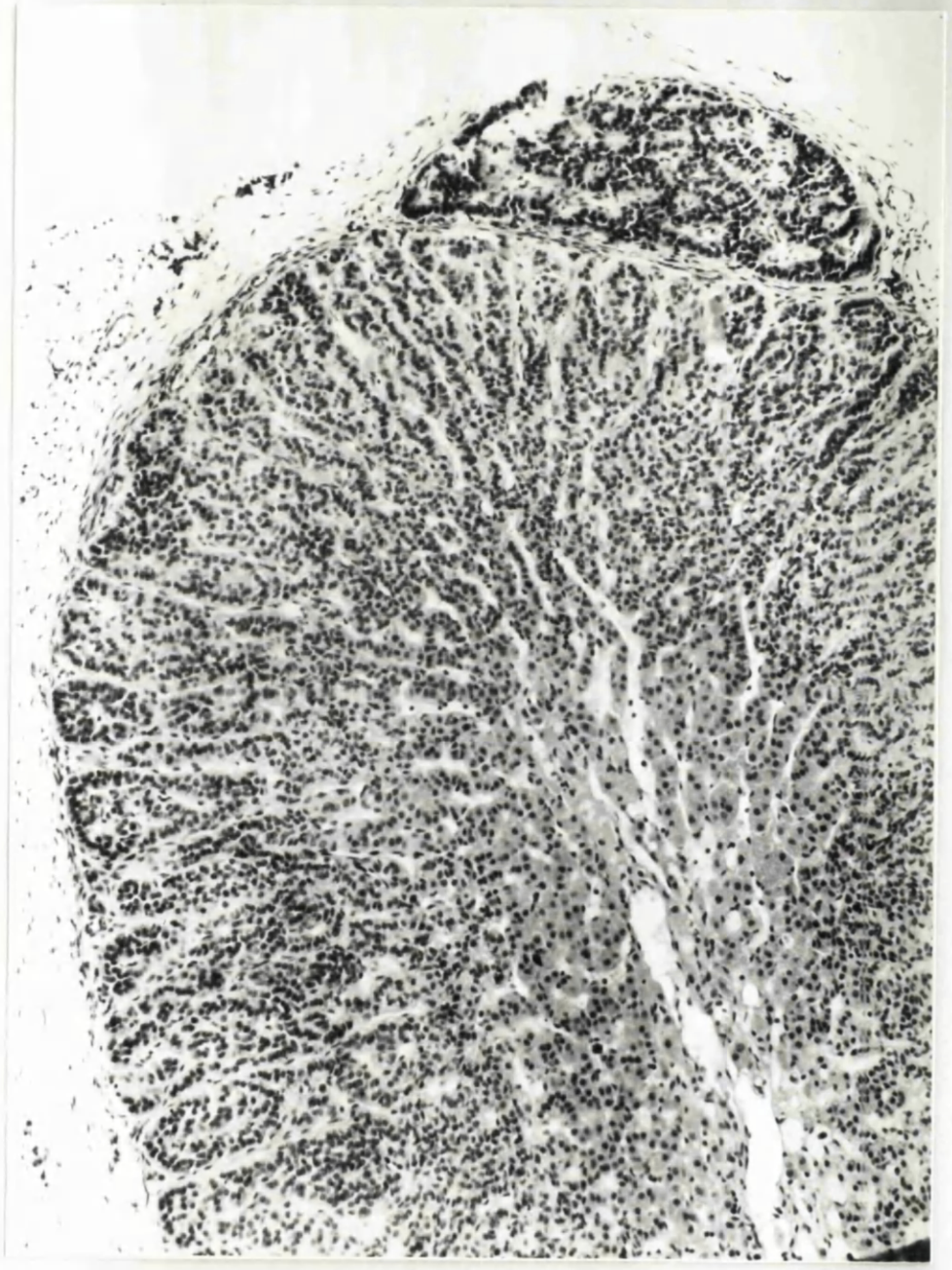


Plate II. Adrenal gland of a newborn anencephalic infant.

H. & E. (X 140).

1968). Johannisson interpreted the marked accumulation of osmiophilic droplets in the cytoplasm of the adrenocortical cells of these infants as further evidence of lack of secretory activity.

The trophic stimulus to the human foetal adrenal glands.

The apparently normal development of the adrenal glands of anencephalic foetuses during the first half of pregnancy suggests that a functioning foetal pituitary gland is not necessary for the establishment of the foetal adrenal gland or for the early development of the foetal zone. Angevine (1938) found no relationship between foetal pituitary size and the state of the adrenals and suggested that foetal zone atrophy in anencephaly is more a result of cerebral and neurohypophyseal maldevelopment. Bloch & Benirschke (1956) have quoted Meyer (1912), "it has been our experience that the premature involution of the foetal zone is not unique to the anencephalic but may be associated with such anomalies as hydrocephaly, hemianencephaly and cephalomeningocele, provided that there is destruction of or a reduction in the size of the hypothalamus". In a study of twenty hydrocephalic infants, Benirschke (1956) observed foetal zone atrophy in only five cases, each of

which evidenced a lack of a normally functioning hypothalamo-neurohypophyseal system.

Bloch & Benirschke (1956) proposed that the foetal zone is maintained during the first half of pregnancy by human chorionic gonadotrophin (HCG) from the placenta. The production of this hormone, in terms of placental weight, falls markedly between ten and twenty weeks of pregnancy **(Selenkow,** Saxena, Dana & Emerson, 1969). Luteinizing hormone (LH), which has been detected in the foetal pituitary, using bioassay techniques (Levina, 1968), by thirteen to fourteen weeks and, by staining of secretory granules (Conklin, 1968), by twenty-two weeks of pregnancy, has been suggested as the stimulus to the foetal zone during the second half of gestation (Bloch & Benirschke, 1956). They further suggested that the reduction in the circulating oestrogen levels which follows placental separation at birth would result in diminished LH secretion by the foetal pituitary causing foetal zone involution. Lanman (1962), however, favoured adrenocorticotrophin as the trophic stimulus to the foetal zone. He administered ACTH to an anencephalic infant for fifteen days, after which the adrenal glands were as large as those of a normal newborn infant. The absence of a description of the histological

appearance of these glands and the failure by Klevit (1966) to detect any increase in urinary steroid excretion by an anencephalic infant treated with ACTH, make it difficult to assign to this hormone with any certainty, a role of foetal zone stimulation. Johannisson (1968) studied the ultrastructure of the adrenal glands of human foetuses treated with ACTH, HCG or an anti-HCG serum and observed distinct, but different, changes with each of these substances in the electron microscopic appearance, which she interpreted as indicating stimulation of steroidogenic activity by both hormones. The occurrence of a similar pattern of foetal zone involution in accessory adrenocortical nodules to that in the main gland (Lewis & Pappenheimer, 1916) suggests the influence of some systemic factor on the maintenance of the foetal zone. Its nature and origin, however, can at present be only matters for speculation, although there is strong evidence for the mediation of some foetal pituitary factor during the second half of gestation.

The steroidogenic function of the human foetal adrenal gland.

The peculiar morphology and post-natal involution of the human foetal adrenals has no doubt promoted a large part

of the extensive investigation undertaken in the past twenty years to elucidate their steroidogenic capacity during pregnancy and in the peri-natal period. Study has, however, been hindered by the absence of a suitable experimental animal. Lanman (1953), in a comprehensive review of foetal adrenal morphology and comparative anatomy, reported that the presence of a foetal zone is restricted to primates, edentata and the larger felidae. It is not known, however, if the foetal zone of these species displays the same pattern of post-natal involution as occurs in man. Sucheston & Cannon (1968) have reviewed reports of the occurrence in the adrenal cortex of several mammals of a developmental zone (e.g. the X-zone of the newborn mouse). These zones have in common: (1) large polyhedral cells with acidophilic cytoplasm and indistinct cell boundaries (2) location between the maturing cortex and the medulla (3) disappearance as an entity as the cortical material assumes its permanent pattern. The authors emphasize that this zone may appear during the foetal or post-natal period in different mammals, and that it is unknown whether it represents a single functional unit in all species. Use of human foetal material has restricted investigators to the study of adrenal glands obtained from, in the main,

the first half of pregnancy. More mature material is most often obtained in cases of congenital abnormality or still birth. A lack of knowledge exists, therefore, concerning the function of the adrenal gland of the human foetus in the later stages of pregnancy.

The earliest studies of steroidogenesis in the human foetal adrenal gland involved the chromatographic identification of steroids isolated from adrenal extracts of the foetus and neonate. Staemmler (1953) isolated corticoids from the adrenal glands of foetuses from twenty weeks of gestation until birth. The presence of androstenedione in the adrenal glands of a foetus of thirty-one weeks gestation and a newborn male pseudohermaphrodite was reported by Bloch, Benirschke & Dorfman (1955). Androstenedione, 11β-hydroxyandrostenedione, 11-oxoandrostenedione and androstanedione were isolated from adrenal extracts of foetuses of nine to twenty-one weeks gestation by Bloch, Benirschke & Rosemberg (1956). The presence of cortisol in the glands of the older foetuses which they studied is consistent with the report by Gardner & Tice (1957) of the isolation of this steroid from adrenal glands of newborn infants of five and a half months of gestation until full-term.

Subsequent to these early indications of steroidogenic

potential, the results of numerous in vitro studies have demonstrated the capacity of the human foetal adrenal glands for most, if not all, the steroid transformations effected by the adult gland (see Table 1). Bloch & Benirschke (1959, 1962) demonstrated the de novo synthesis of C_{19} steroids and cortisol from acetate by slices of adrenal glands from foetuses of twelve to twenty-one weeks gestation. Pregenolone was converted to several C_{19} and C_{21} steroids by adrenal homogenates from foetuses obtained as early as six to eight weeks of gestation (Cooke, Vanha-Perttula & Klopper, 1968). The foetal adrenal gland displays a high in vitro activity of steroid sulphokinases. Klein & Giroud (1965, 1966, 1967) have reported the formation of C-21 sulphates of various corticosteroids by slices of adrenal glands of newborn infants, and Jaffe, Pérez-Palacios, Lamont & Givner (1968) the de novo synthesis of Δ^5-3β-hydroxysteroid sulphates from acetate. The foetal adrenal possesses the capacity to hydroxylate the steroid nucleus in vitro at the 6β-, 7-, 11β-, 16α-, 17α-, 18-, 20α-, 20β- and 21- positions.

Some authors have reported a low activity of the 3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) and/or steroid Δ^4- Δ^5 isomerase (EC 5.3.3.1) enzymes in foetal

Table 1. Steroids isolated from human foetal adrenal glands following incubations with steroid precursors.

Substrate*	Metabolite
Acetate (1 - 3)+	Cholesterol, Δ^5P, DHA, 17$\alpha\Delta^4$P, F, Δ^4A, 11$\beta\Delta^4$A; cholesterol-S, Δ^5PS, DHAS.
20α-cholesterol (4)	17α,20α-cholesterol, DHA, B, F, Δ^4A.
Δ^5P (5 - 14)	17α,21Δ^5P, 17$\alpha\Delta^5$P, 21Δ^5P, 16$\alpha\Delta^5$P, DHA, 16αDHA, 17$\alpha\Delta^4$P, Δ^4P, DOC, S, B, F, Δ^4A, 11$\beta\Delta^4$A, T, Δ^5PS, 17$\alpha\Delta^5$PS, DHAS.
DHA (10, 15 - 17)	7-DHA, Δ^4A, 11$\beta\Delta^4$A, T, DHAS.
Δ^4P (5, 6, 9, 13, 14; 16, 18 - 24)	16$\alpha\Delta^4$P, 17$\alpha\Delta^4$P, 11$\beta\Delta^4$P, 11β,17$\alpha\Delta^4$P, 17α,20$\beta\Delta^4$P, DOC, B, S, F, 16αF, 11-dehydroB, aldosterone, Δ^4A, 11$\beta\Delta^4$A, DOCS, SS.
DOC (24)	DOCS, BS.
B (24)	BS.
S (24)	SS, FS.
T (14)	6βT, 11βT, 16αT, Δ^4A, 11$\beta\Delta^4$A.
Δ^5PS (25)	17$\alpha\Delta^5$PS, DHAS, 16αDHAS.
17$\alpha\Delta^5$P (10)	DHA, 16αDHA, Δ^4A, F, 17$\alpha\Delta^5$PS, DHAS, 16αDHAS.
DHAS (10)	DHA, Δ^4A.
E_2-17β (26)	E_3.

Table 1 contd.

* Trivial names and abbreviations of steroids used in the present study are listed on page xi.

* References

1., 2. Bloch & Benirschke (1959, 1962); 3. Jaffe, Pérez-Palacios, Lamont & Givner (1968); 4. Shimizu, Shimao & Tamaka (1965); 5. Klein & Giroud (1967); 6. Longchampt & Axelrod (1964); 7. Matsumoto, Endo, Yamane, Kurachi & Uozimi (1968); 8. Villee (1967); 9.,10. Villee & Loring (1965, 1969); 11. Pasqualini, Lowy, Albepart, Wiqvist & Diczfalusy (1970); 12. Cooke, Vanha-Perttula & Klopper (1968); 13. Villee (1966); 14. Villee & Driscoll (1965); 15. Sulcova, Capkova, Jirasek & Starka (1968); 16. Bloch, Tissenbaum, Rubin & Deane (1962); 17. Jirasek, Sulcova, Capcova, Röhling & Starka (1969); 18. Solomon, Lanman, Lind & Lieberman (1958); 19. Charreau, Dufau, Villee & Villee (1968); 20. Klein & Giroud (1966); 21. Dufau & Villee (1969); 22. Hillman, Stachenko, & Giroud (1962); 23. Bloch, Romney, Klein, Lipiello, Cooper & Goldring (1965); 24. Klein & Giroud (1965); 25. Pérez-Palacios, Pérez & Jaffe (1968); 26. Nakayama, Arai, Satoh, Nagotomi, Tabei & Yanihara (1966).

adrenal glands obtained during the first half of pregnancy (Bloch & Benirschke, 1962; Villee & Villee, 1965; Cooke, Vanha-Perttula & Klopper, 1968). Bloch, Tissenbaum, Rubin & Deane (1962), however, obtained comparatively large conversions of DHA to androstenedione by adrenal glands from foetuses of nine to nineteen weeks gestation. Androstenedione was the major product of pregnenolone metabolism by homogenates of the adrenal glands of a newborn hydrocephalic infant (Villee & Loring, 1965).

The steroid endocrinology of the foeto-placental unit.

Davis & Plotz (1956) suggested a close functional relationship between the maternal and foetal adrenal glands and the placenta. This was followed in 1964 by the formulation by Diczfalusy of the concept of a foeto-placental unit. He proposed that the foetus and placenta represent incomplete steroidogenic systems, each lacking essential enzyme activities which are present in the other. The foetus and the placenta together, therefore, arecapable of elaborating biologically active steroids, either by de novo synthesis or from circulating maternal precursors. The groups of Diczfalusy, in Stockholm, and Solomon, in Montreal, have since made valuable contributions to the understanding

of the role of the foetal adrenal gland within the foeto-placental unit at midgestation. Their experimental approach has entailed: (1) perfusion of intact previable foetuses, adrenalectomized foetuses, placentas and the intact foeto-placental unit (2) injection into the foeto-placental circulation and uterine arteries (3) infusion into the amniotic fluid and ante-cubital vein of radioactive precursors. Metabolites isolated from the foetal adrenal following such experiments are listed in Table 2. The presence of a steroid in a particular tissue, while indicative of its synthesis in that tissue, might however, result from transport in the circulation and does not, therefore, represent unequivocal proof of its synthesis at that site.

The metabolism of steroids within the foeto-placental unit at mid-pregnancy has recently been reviewed by Diczfalusy (1969). The placenta does not convert perfused acetate to cholesterol nor cholesterol to C_{19} steroids, whereas the foetal adrenal appears to represent a quantitatively important site of cholesterol metabolism and perhaps its biosynthesis, at midgestation (Telegdy, Weeks, Wiqvist & Diczfalusy, 1970). The adrenal gland of the perfused pre-viable foetus possesses a high activity of the enzymes involved in the formation of the 3-sulphates of Δ^5-3β-hydroxysteroids (Bolté, Wiqvist

Table 2. Steroids isolated from human foetal adrenal glands following perfusion experiments with radioactive precursors.

Substrate*	Metabolite
Acetate (1 - 3)+	Cholesterol, Δ^5P, 17$\alpha\Delta^5$P, DHA, Δ^4P, Δ^4A.
Cholesterol (3,4)	Δ^5P, 17$\alpha\Delta^5$P, DHA, Δ^4P.
Δ^5P (5)	Δ^5PS, 17$\alpha\Delta^5$PS, DHAS, DHA.
17$\alpha\Delta^5$P (6 - 8)	17$\alpha\Delta^5$PS, DHAS, DHA, 17$\alpha\Delta^4$P, F.
21Δ^5P (9)	DOC, B.
17α,21Δ^5P (10)	DHA, F.
16$\alpha\Delta^5$P (7)	16α,20$\alpha\Delta^5$P, 16$\alpha\Delta^4$P.
DHA (11)	DHAS.
Δ^4P (12 - 16)	16$\alpha\Delta^4$P, 17$\alpha\Delta^4$P, Δ^4A, 11$\beta\Delta^4$A, 3αB, 20$\beta\Delta^4$P, 20$\alpha\Delta^4$P, DOC, B, F, 3α,20α P, DOCS, BS.
17$\alpha\Delta^4$P (8,17)	17α,20$\alpha\Delta^4$P, F, T, Δ^4A.
Δ^4A (18 - 20)	11$\beta\Delta^4$A, 11βT, T, TS.
T (18 - 20)	11$\beta\Delta^4$A, 11βT, Δ^4A, TS.
B (21,22)	aldosterone, 11-dehydroB, BS.
3α,20αP (23)	3α,20αPS.
E_3-16G (24)	E_3-3S,16G.

* Trivial names and abbreviations of steroids used in the present study are listed on page xi.

Table 2 contd.

+ References

1. Solomon (1967); 2. Telegdy, Weeks, Lerner, Stakemann & Diczfalusy (1970); 3. Telegdy, Weeks, Archer, Wiqvist & Diczfalusy (1970); 4. Coutts & MacNaughton (1969); 5. Solomon, Bird, Ling, Iwamiya & Young (1967); 6. Pion, Jaffe, Wiqvist & Diczfalusy (1967); 7. Reynolds, Wiqvist & Diczfalusy (1969); 8. Jackanicz, Wiqvist & Diczfalusy (1969); 9. Pasqualini, Lowy, Albepart, Wiqvist & Diczfalusy (1970); 10. Pasqualini, Lowy, Wiqvist & Diczfalusy (1968); 11. Boltè, Wiqvist & Diczfalusy (1968); 12. Bird, Wiqvist, Diczfalusy & Solomon (1966); 13. Zander, Holzmann, von Münstermann, Runnebaum & Siler (1969); 14. Greig & MacNaughton (1967); 15. Diczfalusy (1967); 16. Bird, Solomon, Wiqvist & Diczfalusy (1965); 17. Younglai, Stern, Ling, Leung, & Solomon (1969); 18. Mancuso, Benagiano, Dell'Acqua, Shapiro, Wiqvist & Diczfalusy (1968); 19. Benagiano, Kincl, Zielske, Wiqvist & Diczfalusy (1967); 20. Benagiano, Mancuso, Mancuso, Wiqvist & Diczfalusy (1968); 21. Pasqualini, Wiqvist & Diczfalusy (1966); 22. Pasqualini, Mozere, Wiqvist & Diczfalusy (1969); 23. Cooke, Wiqvist & Diczfalusy (1969); 24. Goebelsmann, Eriksson, Diczfalusy, Levitz & Condon (1966).

& Diczfalusy, 1966; Solomon, Bird, Ling, Iwamiya & Young, 1967); the 21-sulphates of corticosteroids (Bird, Solomon, Wiqvist & Diczfalusy, 1965) and steroid 17β-sulphates (Benagiano, Mancuso, Mancuso, Wiqvist & Diczfalusy, 1968). The placenta, in contrast, is unable to sulphoconjugate steroids (Bolté, Mancuso, Eriksson, Wiqvist & Diczfalusy, 1964; Levitz et al., 1967) but possesses a very high activity of steroid sulphatases (Lamb, Mancuso, Dell'Acqua, Wiqvist & Diczfalusy, 1967) which appear to be absent from foetal tissues (Bolté et al., 1966). The foetal adrenal is very active in the removal of the side-chain of Δ^5-3β-hydroxysteroids (Pion, Jaffe, Wiqvist & Diczfalusy, 1967; Pasqualini, Lowy, Wiqvist & Diczfalusy, 1968) but possesses only a limited ability to effect this transformation with Δ^4-3-oxosteroids (Solomon et al., 1967). The placenta appears incapable of side-chain cleavage of either group of steroids (Pion, Jaffe, Eriksson, Wiqvist & Diczfalusy, 1965). The foetal adrenals extensively hydroxylate perfused steroids. The hydroxylating ability of the placenta appears, however, to be restricted to the 6β-position (Kitchin, Pion & Conrad, 1967). Perfusion with progesterone of adrenalectomized foetuses (Wilson, Bird, Wiqvist, Solomon & Diczfalusy, 1966) and of intact foetuses previously treated with the 11β-

hydroxylase inhibitor, metyrapone, suggested that the adrenals are the sole site of synthesis of 11β- and 21- but not 17α- or 6β-hydroxylated metabolites.

Biogenesis of oestrogens within the foeto-placental unit.

Pregnancy is characterized by a marked rise in the maternal urinary excretion of oestrogens, especially oestriol, (Brown, 1956; Diczfalusy & Lauritzen, 1961; Jayle, 1965). Since removal of the placenta, but not the maternal adrenals or ovaries, resulted in a rapid fall in urinary oestrogen excretion to the non-pregnant levels, the placenta was long considered the major source of these compounds. Cassmer (1959), however, showed that interruption of the foeto-placental circulation by ligation of the umbilical cord resulted in a marked drop in oestrogen excretion, suggesting that the foetus might supply precursors for placental oestrogen synthesis. The foetal adrenal is now considered to be the quantitatively most important source of oestrogen precursors during pregnancy, (Diczfalusy, 1967). The foetal liver, which possesses a limited capacity for the aromatization of Δ^4-3-oxosteroids (Mancuso et al., 1968), is the principal, if not exclusive site of 16α-hydroxylation of oestrone, DHA and their 3-sulphates

(Diczfalusy & Mancuso, 1969).

3β-Hydroxysteroid dehydrogenase isomerase activity in the foeto-placental unit.

The foetal adrenal at mid-term does not convert perfused pregnenolone or DHA into the corresponding Δ^4-3-oxosteroids (Bolté et al., 1966; Solomon et al., 1967), suggesting a functional lack of the 3β-hydroxysteroid dehydrogenases and/or isomerases. The conversions of 17α-hydroxypregnenolone (Jackanicz, Wiqvist & Diczfalusy, 1969), 21-hydroxypregnenolone (Pasqualini, Lowy, Albepart, Wiqvist & Diczfalusy, 1970) and 17α, 21-dihydroxypregnenolone (Pasqualini, Lowy, Wiqvist & Diczfalusy, 1968) into Δ^4-3-oxosteroids might indicate the existence of substrate-specific enzymes in the foetal adrenal at midpregnancy. The apparent absence of significant 3β-hydroxysteroid dehydrogenase isomerase activity from the foetal tissues at mid-term is in contrast to the high activities of this enzyme system displayed by placental tissue (Pion et al., 1965; Dell'Acqua et al., 1967; Reynolds et al., 1968).

The results of perfusion experiments are in broad agreement with those obtained by in vitro techniques. There are, however, instances of inconsistency between the two

approaches (e.g. with respect to 3β-hydroxysteroid dehydrogenase isomerase activities) concerning the quantitative significance of the various pathways which have been established.

The adult and foetal zones of the human foetal adrenal gland.

Little information exists concerning the relative contributions of the zonal tissues of the human foetal adrenal gland to steroidogenesis within the foeto-placental unit. The adrenal gland of anencephalic infants has been suggested as a possible source of adult zone tissue for biochemical investigation (Shahwan, Oakey & Stitch, 1968a). These glands, however, invariably contain some foetal zone cells and the adult zone displays ultrastructural differences from that of the normal foetus. Steroids isolated following incubations of adrenal tissue of anencephalic infants with radioactive precursors are listed in Table 3. These glands appear to display qualitatively similar patterns of steroid metabolism to those observed in normal foetal adrenal tissue. The urinary oestrogen excretion of women pregnant with anencephalic foetuses is only one tenth of that in normal pregnancies (Frandsen & Stakemann, 1961). The foetal zone of the foetal adrenal gland has, therefore, been suggested as the chief source of foetal steroid

Table 3. Steroids isolated following incubations of adrenal glands from newborn anencephalic infants with radioactive steroid precursors.

Substrate*	Metabolite
Δ^5P (1 - 4)+	16$\alpha\Delta^5$P, DHA, 16αDHA, Δ^4P, 11$\beta\Delta^4$P, 16$\alpha\Delta^4$P, 17$\alpha\Delta^4$P, 20$\alpha\Delta^4$P, DOC, B, S, F, Δ^4A, 11$\beta\Delta^4$A, T, Δ^5PS, DHAS.
Δ^4P (2,3)	11$\beta\Delta^4$P, 17$\alpha\Delta^4$P, 20$\alpha\Delta^4$P, DOC, B, S, F, Δ^4A, 11$\beta\Delta^4$A, T.
DHA (5,6)	Δ^4A, 11$\beta\Delta^4$A, T, DHAS.
DHAS (6)	DHA, 16αDHA, Δ^4A, 11$\beta\Delta^4$A.

* Trivial names and abbreviations of steroids used in the present study are listed on page xi.

+ References

1. Cooke (1968); 2 - 5. Shahwan, Oakey & Stitch (1969a,b; 1968a,b); 6. Villee & Loring (1969)

precursors for placental oestrogen synthesis. The changing patterns of urinary steroid excretion in the first few weeks of life have been attributed to foetal zone involution and adult zone development during this period (Mitchell, 1967; Reynolds, 1969).

More direct evidence for zonal function has been obtained by the in vitro incubation of adult and foetal zone fractions, prepared by dissection of slices of foetal adrenal glands, with steroid precursors. Solomon, Lanman, Lind & Lieberman (1958) found both tissue fractions to be capable of converting progesterone to 17α-hydroxy-progesterone and androstenedione with approximately equal efficiency. The conversion of acetate to C_{19} steroids and cortisol by foetal zone fractions was demonstrated by Bloch & Benirschke (1962) who observed differences in the relative capacities of the adult and foetal zone fractions for DHA and androstenedione formation. Hillman, Stachenko & Giroud (1962) reported greater conversion of progesterone to cortisol by foetal than by adult zone fractions. Both zones appeared, therefore, capable of steroidogenesis in vitro and displayed qualitatively similar patterns of steroid metabolism. The adult zone fractions employed in these experiments were, however, due to the paucity of

this tissue, always contaminated by capsular and foetal zone cells.

The activity of 3β-hydroxysteroid dehydrogenase in adult and foetal zone cells, has been investigated histochemically. Some workers have detected a higher activity of this enzyme in the adult than in the foetal zone (Niemi & Baillie, 1965; Goldman, Yakovac & Bongiovanni, 1964). Cavallero & Magrini (1966), however, obtained no formazan deposition in the adrenal gland of foetuses prior to the eighteenth week of gestation and detected activity in the adult, but not the foetal zone, of older foetuses.

The present study.

The microtechnique of Grunbaum, Geary & Glick (1956) was applied in the present study to the preparation of histologically-defined tissue from the adrenal glands of previable human foetuses and newborn infants. This technique permits histological monitoring of tissue sections used for biochemical investigation and was first applied to steroid biochemistry by Griffiths & Glick (1966) in a study of 11β-hydroxylase activity in the adrenal gland of the adult rat.

The substrate in all incubations of the present experiments was dehydroepiandrosterone (DHA). This steroid has been isolated in the free and sulphoconjugated form from the adrenal gland of the human foetus and newborn infant (Matsumoto, Endo, Yamane, Kurachi & Uozimi (1968) and is extensively metabolized in the adrenal gland when perfused into the previable human foetus (Bolté et al., 1966). DHA was chosen as substrate partly because of the limited number of biochemical transformations which it may undergo as compared with, for example, C$_{19}$-Δ^5-3β-hydroxysteroids, and because its availability in a tritiated form of high specific radioactivity makes it suitable for microbiochemical studies.

Isotopically-labelled steroids isolated following incubation of [7α-^{3}H] DHA with adrenal tissue in the present experiments were identified and characterized according to the principle of reverse isotope dilution outlined by Berliner & Salhanick (1956), employing techniques of thin-layer chromatography and derivative formation similar to those described by Griffiths, Grant & Whyte (1963). Quantitation of steroids was achieved by spectrophotometric analysis and gas-liquid chromatography and measurement of radioactivity

by liquid scintillation spectrometry.

The conversion of DHA to DHA sulphate, androstenedione and, in some cases, 11β-hydroxyandrostenedione was studied in (1) whole tissue preparations of the adrenal glands of previable human foetuses and newborn infants; (2) adult and foetal zone fractions (each uncontaminated by cells of the other zone) prepared from the adrenal glands of a foetus of 22 weeks gestation and a newborn hydrocephalic infant; (3) adult zone tissue from the adrenal glands of an anencephalic infant delivered by Caesarean section after 38 weeks of gestation and a newborn anencephalic infant delivered per vaginam after 42 weeks of gestation. The quantitative histological distribution of DHA sulphokinase (EC 2.8.2.1) and 3β-hydroxysteroid dehydrogenase-isomerase activities was determined in the adrenal glands of five previable human foetuses. The effects on the activities of these enzyme systems of variation of the length of the incubation period, the tissue:steroid ratio and the co-factor content of the incubation medium were also investigated.

The results of the present experiments are discussed in relation to those of previous <u>in vitro</u> studies, perfusion experiments of the isolated foetus and the intact foet-

placental unit, histochemical investigations, the ultrastructure of the foetal adrenal gland at different stages of gestation and steroid concentrations in the tissues and body fluids of the foetus and pregnant woman.

EXPERIMENTAL

Chemicals. Nucleotides were purchased from Boehringer & Soehme Gmbh., Mannheim, W. Germany, and glucose-6-phosphate from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Other reagents (unless otherwise stated) were Analar grade purchased from British Drug Houses Ltd., Poole, Dorset. Non-labelled steroids, with the exception of 11β-hydroxytestosterone and its acetate which were obtained from the M.R.C. Steroid Reference Collection, were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., Steraloids Ltd., Croydon, Surrey and Organon Laboratories, Newhouse, Lanarkshire.

Ethanol and methanol (Burroughs A.R. grade), toluene, acetone, diethyl ether and tert.-butanol were used without further purification. Benzene and methylene chloride were passed through a column containing silica gel and conc. H_2SO_4, washed with water, dried over $CaCl_2$ and distilled twice. Other solvents were washed with water or dil. alkali, dried and distilled before use.

Radioactive DHA. [7α-^{3}H] DHA was obtained from Radiochemical Centre, Amersham, Bucks. (500 mCi/mmole) and New England Nuclear Corp., Boston, Mass., U.S.A. (12.9 Ci/mmole)

and stored at 4°C in benzene-methanol (1:5, v/v) at concentrations of 40 μCi/ml and 5 μCi/ml respectively. $[^3H]$ DHA was purified immediately before use by thin-layer chromatography (t.l.c.) in system I (see Table 4). Radioactivity was detected using a radiochromatogram scanner and the area corresponding in mobility to authentic DHA standards eluted. On rechromatography in system I or VI, the $[^3H]$ DHA gave one peak of radioactivity with a Gaussian distribution.

Adrenal tissue. Adrenal glands were obtained from human foetuses of 11 - 24 weeks gestation, a newborn hydrocephalic infant, a newborn post-mature anencephalic infant and an anencephalic foetus delivered by Caesarean section after 38 weeks gestation. The glands were removed within 30 min. of death and maintained at 0 - 4°C until incubation. A small portion of each gland was retained for histological examination.

Preparation of histologically-defined tissue sections.

Fresh-frozen tissue sections were prepared as described by Grunbaum, Geary & Glick (1956). The adrenal glands were stripped of adhering fat, frozen in solid CO_2, taking care to preserve the tripartite

structure of the glands, and transferred to a cryostat at - 15°C. Cylinders of frozen tissue were bored from the glands using a set of stainless steel borers (1.5 - 3.0 mm diameter) and a small electrically-driven press (Barr & Stroud Ltd., Glasgow). The tissue borer assembly is shown in Fig. 1. A borer (a) of suitable size was attached to the motor shaft of the drill press through the adaptor (b). The frozen tissue was placed on dental impression wax on the table of the press which was manually raised until the slowly rotating borer had passed through the gland. The borer was then attached to its support rod (c) by the set screw (d) and mounted on the spherical holder (e) which was clamped in the socket of a Minot rotary microtome. Glands were bored, whenever possible, through an ala rather than a crest (Dobbie & Symington, 1966), in order to obtain the most satisfactory sections (see Fig. 2. and Results section below). After careful alignment on the microtome to ensure section cutting normal to the long axis of the tissue cylinder, 1 - 2 mm of tissue was exposed from the end of the borer and 16 μm thick sections prepared using an anti-curling device similar to that described by Coons, Leduc & Kaplan (1951) (see Fig. 3). The first and then every fourth section was brushed on to

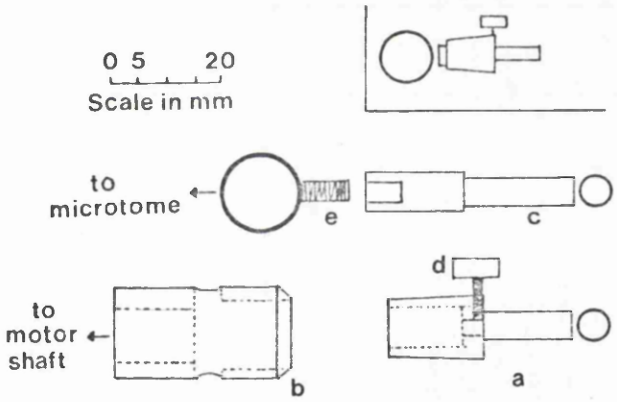


Figure 1. Tissue borer assembly.

(From Grunbaum et al., 1956)

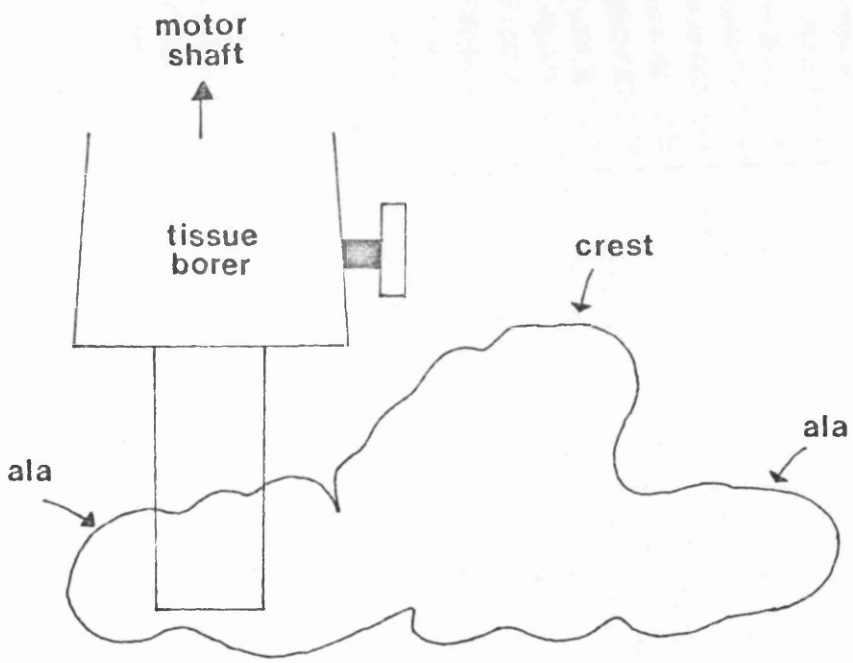


Figure 2. Preparation of fresh-frozen tissue cylinders.

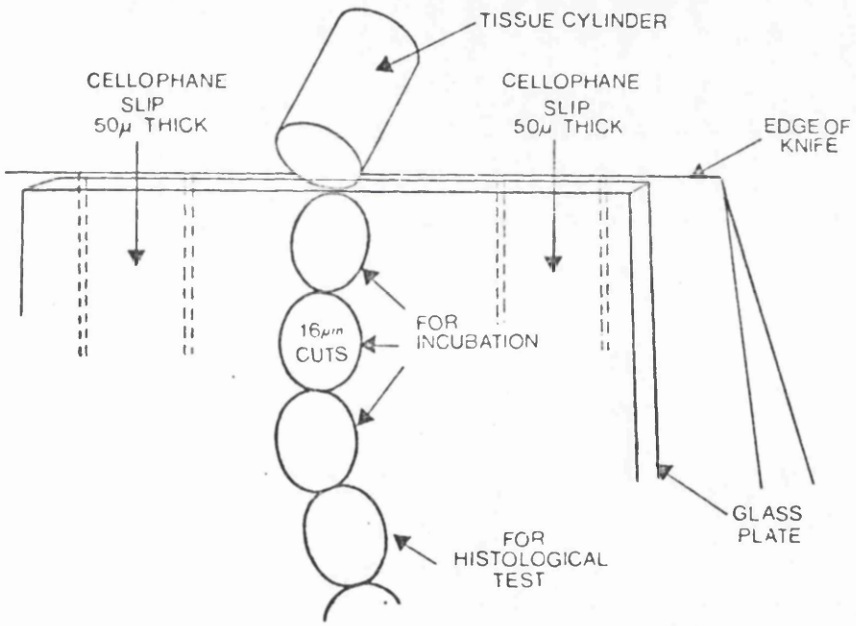


Figure 3. Use of anti-curling device in sectioning fresh-frozen tissue.

a glass microscope slide and the intermediate sections placed on the frozen surface of 10 - 40 µl 0.25M-sucrose containing 0.12M nicotinamide (sucrose-nicotinamide) in a small, glass, stoppered reaction tube (30 mm long, 4 mm internal diameter; Microchemical Specialities Co., Berkeley, Calif., U.S.A.). Examination of the sections on the microscope slides, after staining with haematoxylin & eosin (H. & E.) or toluidine blue (see Appendix I), permitted identification of the zonal tissue contained in each reaction tube.

Whole tissue preparations. Adrenal gland homogenates were prepared in sucrose-nicotinamide using a motor-driven Potter-type homogenizer with a glass mortar and a Teflon pestle. In experiments 4 - 6 & 11, adrenal tissue was finely chopped with a scalpel blade in one drop of sucrose-nicotinamide.

Incubation conditions. The incubation medium, unless otherwise stated, contained 0.05 M-2-amino-2-hydroxymethyl-1,3-propanediol (tris) buffer, pH 7.4, 0.075 M-KCl, 0.03 M-$MgSO_4$, 0.5 mM-NAD, 0.5 mM-NADP, 2.28 mM-ATP, 0.01 M-glucose-6-phosphate and glucose-6-phosphate dehydrogenase

(0.2 Kornberg units/ml.). Tissue:steroid ratios were calculated from the wet weight of tissue in whole tissue incubations and, in the case of incubations with tissue sections, by assuming a specific gravity for adrenal tissue of 1.0 (e.g. for 3 sections of 1.5 mm diameter, 80 µg tissue).

Purified [^{3}H] DHA was dissolved in incubation medium and 50 µl added to each reaction tube using an automatic Hamilton syringe to facilitate speed and reproducibility. The tissue was then disintegrated by "buzzing" (Glick, 1961, 1962) i.e. rapid vibration imparted to the contents by touching the side of the tube against a rapidly rotating rubber stopper, with edges cut to form a pentagon, attached to the shaft of an electric motor.

Unless otherwise stated 1 ml of homogenate was incubated with 2 ml of incubation medium and chopped tissue was suspended in 1 ml sucrose-nicotinamide and incubated with 2.5 ml incubation medium in flasks containing evapourated purified [^{3}H] DHA solution.

Incubations were carried out at 37°C and were terminated after 30 min., unless otherwise stated, by the addition of acetone and cooling to - 15°C. Control

incubations (using boiled tissue) or blank incubations (in the absence of tissue) were included in all experiments.

Experiment 1. A 1% (w/v) homogenate of adrenal tissue from a male foetus of 15 weeks gestation was incubated at tissue:steroid 10,000:1 for 0, 2, 5, 10, 15 and 30 min.

Experiment 2. A 1% (w/v) adrenal homogenate from a male foetus of 17 weeks gestation was incubated at tissue:steroid 10,000:1 for 0, 1, 2, 5, 10, 20, 30, 60 and 120 min. and at tissue steroid 500, 1,000, 2,000, 5,000, 20,000 and 100,000:1 for 30 min. A portion of the adrenal gland was maintained frozen at - 15°C for 2 h before preparation of a 1% (w/v) homogenate and incubation at tissue:steroid 10,000:1 for 30 min. The original homogenate was kept in ice for 90 min and incubated under the same conditions.

Experiment 3. A 5% (w/v) homogenate of adrenal tissue from a female foetus of 15 weeks gestation was incubated at tissue:steroid 1,000, 5,000 and 10,000:1 in normal medium and under nitrogen in medium containing

NAD as the only added co-factor.

Experiment 4. Adrenal gland segments (20 mg) from a female foetus of thirteen weeks gestation were chopped, suspended in 0.4 ml sucrose-nicotinamide and incubated in 1.4 ml medium with and without ATP. After 30 min., the incubation mixtures were transferred to flasks containing [^{3}H] DHA and incubated at tissue:steroid 10,000:1 for a further 30 min. A 10 mg. segment was chopped, suspended in 0.2 ml sucrose-nicotinamide and incubated in 0.8 ml medium containing ATP at tissue: steroid 10,000:1 for 30 min.

Experiment 5. Adult and foetal zone fractions and 28 mg chopped tissue from the adrenal gland of a male foetus of 22 weeks gestation were incubated at tissue: steroid 10,000:1.

Experiment 6. Tissue sections and 9 mg chopped tissue from the adrenal gland of a male foetus of 18 weeks gestation were incubated at tissue:steroid 10,000:1.

Experiment 7. Tissue sections from the adrenal gland

of a male foetus of 24 weeks gestation were incubated at tissue:steroid 5,000:1. A 1% (w/v) homogenate was incubated at tissue:steroid 5,000:1 and 10,000:1.

Experiment 8. Tissue sections and a 1% (w/v) homogenate from the adrenal gland of a female foetus of 15 weeks gestation were incubated at tissue:steroid 10,000:1.

Experiment 9. Tissue sections and a 5% (w/v) homogenate from the adrenal gland of a foetus of 15 weeks gestation were incubated at tissue:steroid 10,000:1 in medium containing no exogenous ATP. The homogenate was also incubated in medium containing added ATP.

Experiment 10. Tissue sections and a 1% (w/v) homogenate from the adrenal gland of a female foetus of 23 weeks gestation were incubated at tissue:steroid 5,000:1 in medium containing no ATP.

Experiment 11. Adult and foetal zone fractions from the adrenal gland of a newborn female hydrocephalic infant were incubated at tissue:steroid 10,000:1. Adrenal segments (70 mg) were chopped and incubated at

tissue:steroid 10,000:1 and 100,000:1.

Experiment 12. Tissue sections and a 1% (w/v) homogenate from the adrenal gland of a newborn female anencephalic infant delivered after 42 weeks gestation were incubated at tissue:steroid 10,000:1 in the presence and absence of added ATP.

Experiment 13. Tissue sections from the adult zone of the adrenal gland of a female anencephalic foetus of 38 weeks gestation were incubated at tissue:steroid 10,000:1. A 1% (w/v) homogenate was incubated at the same tissue: steroid ratio in the presence and absence of added ATP.

Extraction procedures. (a) Whole tissue incubations. Carrier steroids (200-500 µg each of DHA, DHA sulphate, androstenedione and, in some cases, 11β-hydroxyandrostenedione) were added and the flask contents centrifuged. The acetone extracts were removed by aspiration and the protein precipitates washed with acetone (2 x 5 ml). The combined acetone extracts were evapourated to dryness.

(b) Tissue section incubations. The reaction tubes were centrifuged, the acetone extracts

removed, and the protein precipitates washed with acetone (3 x 100 µl). The acetone extracts in experiments 5 and 11 were combined into adult and foetal zone fractions following examination of the sections on the microscope slides, otherwise the contents of each reaction tube were processed separately. Carrier steroids (as above) were added and the acetone extracts evapourated to dryness.

The residues remaining after the evapouration of the acetone extracts from both types of incubation were dissolved in water (5 ml) and extracted with benzene-chloroform (6:1, v/v; 3 x 5 ml). The aqueous fractions were then saturated with $(NH_4)_2SO_4$ and steroid conjugates extracted with ether-ethanol (3:1, v/v; 3 x 5 ml) (Edwards, Kellie & Wade, 1953).

The precipitates remaining in the small reaction tubes were assayed for protein nitrogen by the Nayyar & Glick (1950) modification of the Grief (1950) bromosulphalein binding method (see Appendix II).

Thin-layer chromatography. Chromatographic separations were achieved on thin-layers of Merck silica gel $HF_{254/366}$. The steroids were detected under u.v. light at 254 nm or 366 nm. The solvent systems used are shown in Table 4 and

Table 4. Solvent systems used in the present study for the thin-layer chromatographic separation of steroids.

I	Chloroform-acetone (37:3, v/v)
II	Cyclohexane-ethyl acetate (1:1, v/v)
III	Benzene-ethyl acetate (9:1, v/v)
IV	tert.-Butanol-ethyl acetate-5N-NH_4OH (41:50:20, by vol.)
V	Chloroform-acetone (7:1, v/v)
VI	Chloroform-ethanol (19:1, v/v)

the R_f values of steroids used in the present study listed in Appendix III.

Unconjugated steroids were recovered from silica gel by extraction with ethyl acetate (2 x 5 ml) after addition of water (2 ml), and steroid sulphates with ether-ethanol (3:1, v/v; 2 x 5 ml) after addition of saturated NaCl solution (2 ml).

Derivative formation. (a) Acetylation. Steroids were acetylated as described by Zaffaroni & Burton (1951). Pyridine (5 drops) and redistilled acetic anhydride (5 drops) were added to the dried steroid. The reagents were evapourated under a stream of filtered air after 2 h at 50°C or 16 h at room temperature.

(b) Saponification. Steroid acetates were hydrolysed by a modification (Ward & Grant, 1963) of the method of Neher, **Desaulles,** Vischer, Wieland & Wettstein (1958). The steroid acetates were dissolved in methanol (1 ml) and 2% aq. K_2CO_3 (0.2 ml) added. After 16 h at room temperature, water (2 ml) was added and the steroids extracted with ethyl acetate (2 x 5 ml).

(c) Reduction. Steroids were reduced using a modification of the procedure of Southcott,

Bandy, Newson & Darroch (1956). Ice-cold 0.5% methanolic $NaBH_4$ was added to the chilled, dried steroid. The reaction was stopped after 1 h at 0°C by the addition of glacial acetic acid (1 drop) and the steroids extracted with ethyl acetate (2 x 5 ml) after addition of water (2 ml).

(d) Solvolysis. Steroid sulphates were hydrolysed by the method of Burstein & Lieberman (1958). Sulphated fractions were evapourated to dryness, dissolved in water (5 ml) and the pH reduced to 1.0 with 4 N-H_2SO_4. Following saturation with NaCl, the steroids were extracted with ethyl acetate (2 x 5 ml) and the pooled extracts incubated for 4 h at 50°C. Alternatively, water, adjusted to pH 1.0 with H_2SO_4, was extracted twice with an equal volume of ethyl acetate and 5 ml of the combined extracts incubated with the dried sulphate fractions.

The ethyl acetate incubates were then washed with 5% aq. $NaHCO_3$ (5 ml) and water (2 x 2 ml).

Quantitative estimation of steroids. Δ^4-3-oxosteroids (androstenedione, 11β-hydroxyandrostenedione and their derivatives) were dissolved in ethanol and their extinctions measured in a Unicam SP 500 spectrophotometer. Concentrations were calculated by comparison with the extinctions of

standard solutions or from the molar extinction coefficients.

DHA and its derivatives were estimated by gas liquid chromatography using a Perkin-Elmer model F11 gas chromatograph fitted with a flame ionization detector and 6 ft glass columns packed with 3% SE-30 or 1% OV-1 on 100 - 120-mesh Gas-chrom Q. 1 µl volumes were injected using ethanol, hexane or methylene chloride as solvent. DHA was used as internal standard for DHA acetate and DHA acetate as internal standard for DHA and 5-androstene-3β, 17β-diol. Quantitation was achieved by comparing the peak height ratios of the sample mixtures with those of standard mixtures.

Measurement of radioactivity. Extracts were evapourated to dryness in scintillation vials and dissolved in 8 ml of toluene scintillator containing 3 g/litre of 2,5-diphenyloxazole (PPO) and 0.1 g/litre of 1,4-bis-2 (4-methyl-5-phenyl-oxazoyl)-benzene (dimethyl POPOP). Radioactivity was measured in a Packard Tri-Carb Model 3214 liquid scintillation spectrometer, counting efficiencies being calculated by comparison with a sealed radioactive standard, or in a Beckman Model DPM-100 liquid scintillation spectrometer using external standardization. Counting efficiencies for unquenched samples were approximately 40% and 50% respectively. Aqueous solutions of steroids were dissolved in a mixture

of the above toluene scintillator and ethanol in the ratio 175:25 (v/v). The degree of quenching was calculated either by comparison with the counting efficiency of similarly quenched standards or by use of the external standard.

Thin-layer plates were scanned for radioactivity using a Packard Model 7200 radiochromatogram scanner or a Panax Thin-layer radiochromatogram scanner (detection efficiencies for tritium 0.6% and 1 - 2% respectively).

Identification and characterization of steroids. Steroids isolated from incubations were identified by the principle described by Salhanick & Berliner (1956). After isotope dilution by the addition of relatively much larger amounts of the appropriate unlabelled compounds, steroids were purified by t.l.c. and derivative formation (Griffiths, Grant & Whyte, 1963). Steroids were considered radiochemically pure when the specific activities (disintegrations/min/mole) of the metabolite and one or two of its derivatives were constant ± 5%. The radiochemical purity of some steroids was checked by recrystallization from hexane-acetone after addition of 10 - 20 mg of the appropriate carrier steroid. Percentage conversions of the incubated [^{3}H] DHA to its metabolites were calculated by extrapolation from the

specific activities of the radiochemically pure steroids to the mass of carrier steroid originally added (Percentage conversion = mean specific activity x nmoles carrier added x 100 / disintegrations/min of $[^{3}H]$ DHA incubated).

Conjugated steroid extracts were chromatographed in system IV to remove traces of free steroids (Pierrepoint, 1967), solvolysed and chromatographed in system I. The areas corresponding in mobility to authentic DHA were eluted and their specific activities determined. The solvolysed DHA sulphate was then acetylated and chromatographed in system III before measurement of the second specific activity. The third specific activity in experiments 5 and 11 was obtained after hydrolysis of the DHA acetate, chromatography in system I, reduction to 5-androstene-3β,17β-diol and chromatography in system II.

Benzene-chloroform extracts were chromatographed in system I or V to fractionate the unconjugated steroids. DHA extracts were rechromatographed in system VI prior to purification and specific activity determinations as for the solvolysed DHA sulphate extracts. Androstenedione and 11β-hydroxyandrostenedione extracts were subjected to acetylation procedure and chromatographed in system II before determination of their first specific activities.

The second determinations were made after reduction to testosterone and 11β-hydroxytestosterone respectively and chromatography in system II. In experiments 5, 6, 11 and 12 the third specific activities were obtained after acetylation of the testosterone and 11β-hydroxytestosterone and chromatography in system I.

RESULTS

The adrenal glands obtained from pre-viable human foetuses for use in the present study were histologically normal (c.f. Plate I). The histological appearance of the adrenal tissue obtained from the newborn anencephalic and hydrocephalic infants is described below with the results of the appropriate experiments.

Sections containing adult or foetal zone tissue, uncontaminated by cells of the capsule or of the other zone, were obtained using the technique described. The zonal tissues were readily distinguishable by the higher nuclear:cytoplasmic ratio of the adult zone cells compared to those of the foetal zone (see Plates III & IV). Sections of adult zone free from cells of the foetal zone were rarely obtained if glands were bored through their crests. Bores were, therefore, when possible, taken through the alae of the glands.

With the exception of experiment 11 (see below), 96-103% of the incubated radioactivity was associated with carrier DHA following incubation of [^{3}H] DHA with boiled tissue or in the absence of tissue. Small amounts of radioactivity were invariably associated with carrier

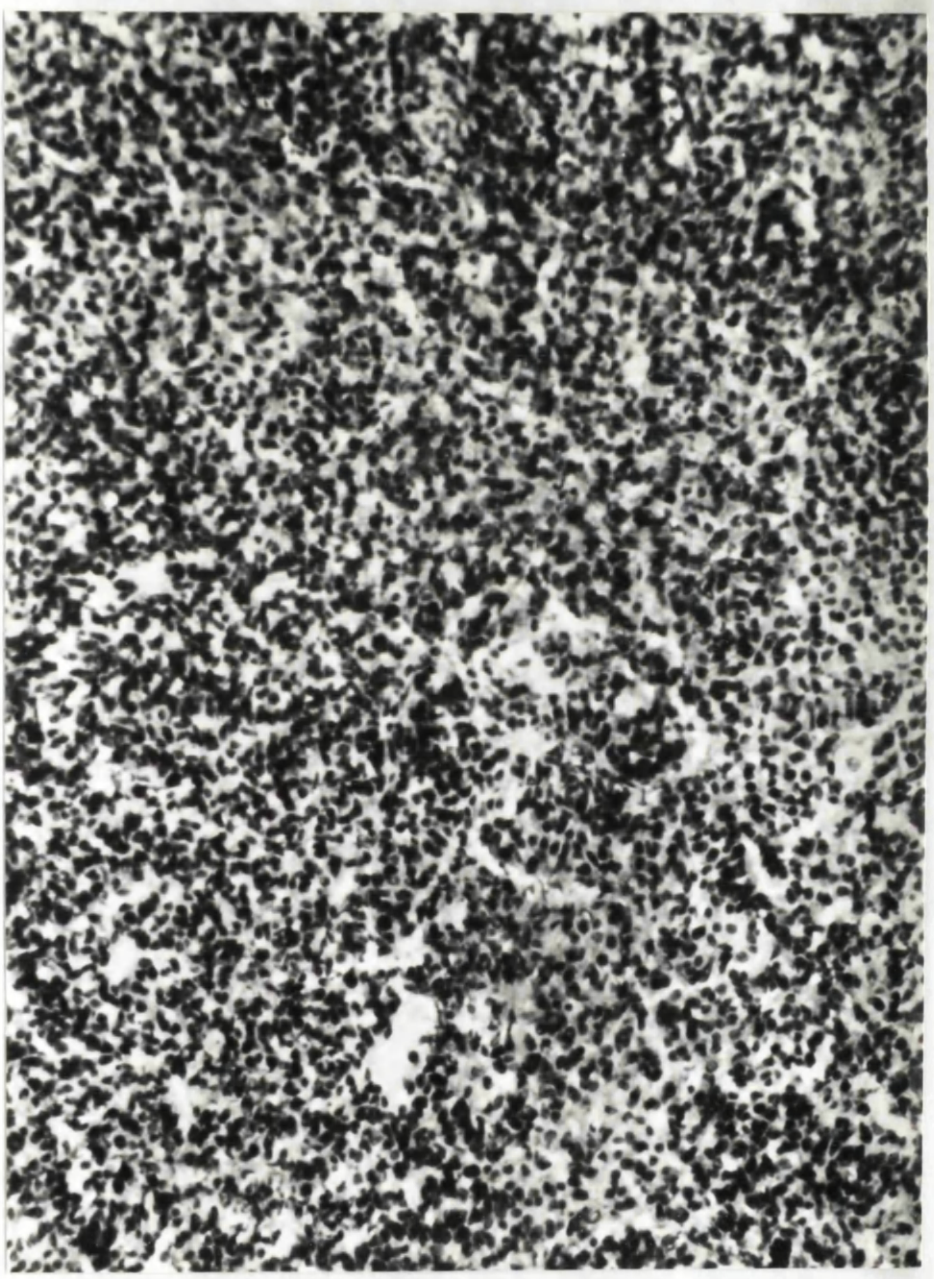


Plate III. Section (16 μm) of adult zone tissue obtained by the microtechnique of Grunbaum *et al.* (1956). H. & E. (X 250).

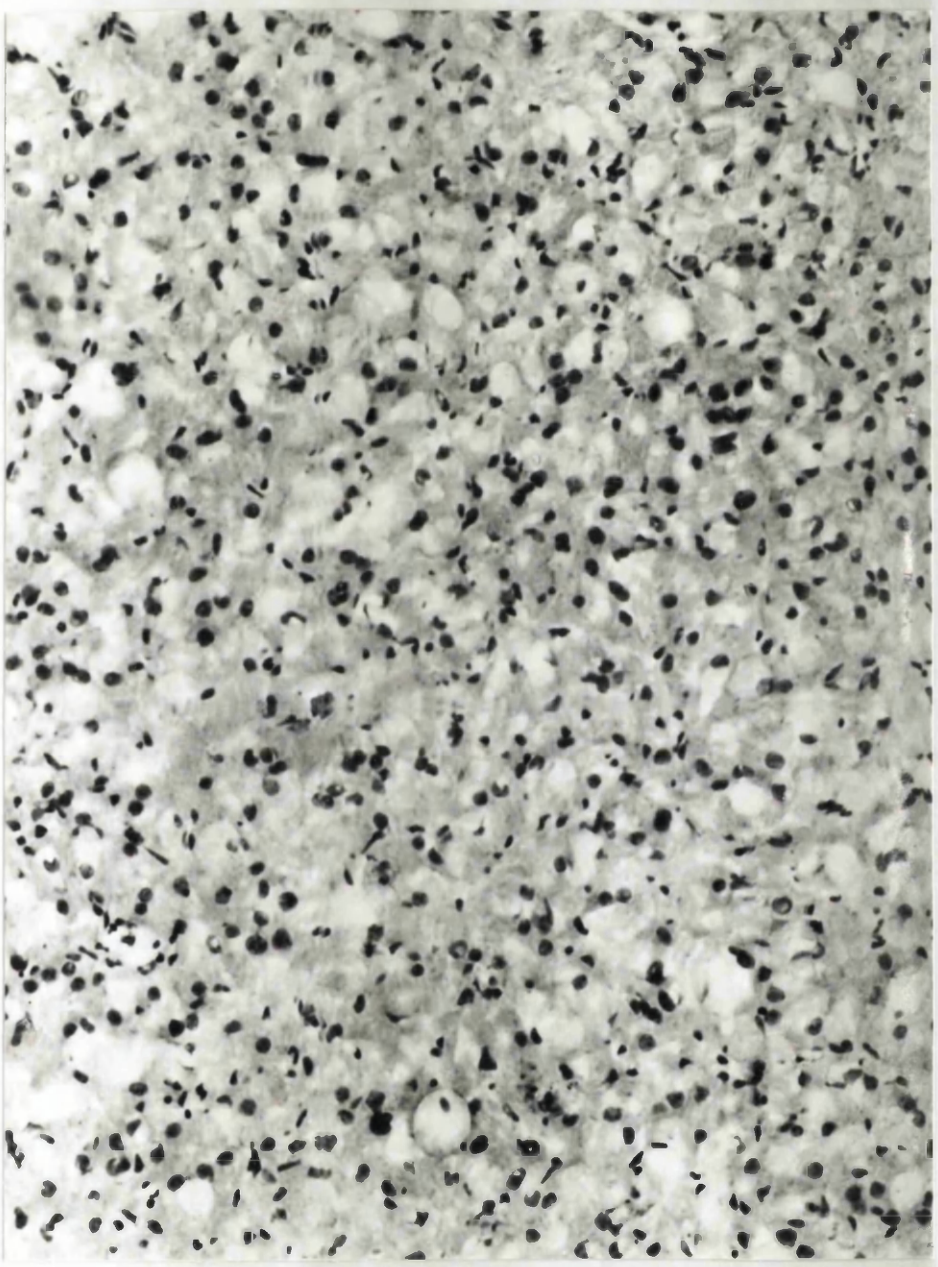


Plate IV. Section (16 µm) of foetal zone tissue obtained by the microtechnique of Grunbaum *et al.* (1956). H. & E. (X 250).

androstenedione and, where investigated, 11β-hydroxyandrostenedione isolated following chromatography of the benzene-chloroform extracts. This radioactivity always dissociated during rechromatography and the subsequent derivative formation.

Evidence is presented in Tables 5 - 17 for the identification by chromatography and derivative formation to constant specific activity of steroids isolated from incubations of [^{3}H] DHA with human foetal and neonatal adrenal tissue. Additional evidence for the radiochemical purity of certain extracts was obtained by recrystallization to constant specific activity (Tables 18 & 19). In experiment 12, the radioactivity present in each extract was measured prior to the addition of further carrier steroid. The percentage conversions calculated from the average specific activities of the crystals were in agreement ± 2% with those calculated after chromatography and derivative formation.

<u>The effect of length of incubation period on [^{3}H] DHA metabolism</u>.

Adrenal tissue from a foetus of 15 weeks gestation

Table 5. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 1)

Steroid isolated and derivative formed	Specific activity (disintegrations/min/nmole)					
	0 min	2 min	5 min	10 min	15 min	30 min
DHA						
DHA	5,201	5,003	2,512	1,762	1,285	889.3
DHAAc	5,113	4,818	2,488	1,693	1,257	866.9
DHAS						
DHA	-	201.8	2,638	3,357	3,863	3,832
DHAAc	-	206.1	2,596	3,319	3,782	3,889
Δ^4A						
Δ^4A	-	1.031	2.327	4.186	6.311	10.84
T	-	0.9855	2.233	4.030	6.043	10.31

Table 6. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 2)

Incubation	Specific activity (disintegrations/min/nmole)					
	DHA		DHAS		Δ^4A	
	DHA	DHAAc	DHA	DHAAc	Δ^4A	T
Blank	5,867	5,863	0	0	0	0
0 min	5,672	5,423	0	0	0	0
1	5,479	5,298	430.1	411.7	2.121	2.016
2	5,034	4,801	976.9	939.8	4.733	4.509
5	3,970	3,785	2,212	2,186	10.87	10.50
10	2,866	2,831	3,890	3,724	16.18	15.91
20	1,527	1,485	5,975	5,880	22.63	21.81
30	496.9	478.2	7,462	7,314	29.77	28.93
60	227.0	217.7	7,717	7,510	50.13	48.10
120	112.3	109.3	7,998	7,605	88.34	85.20
500:1	4,626	4,581	752.3	718.3	117.8	115.1
1,000:1	3,516	3,349	2,900	2,759	51.32	48.84
2,000:1	2,198	2,081	5,920	5,912	40.13	39.07
5,000:1	954.7	940.2	7,001	6,702	33.85	32.19
20,000:1	277.1	268.9	4,000	3,909	12.58	11.98
100,000:1	100.9	97.03	1,631	1,598	2.575	2.449
Unfrozen	499.5	480.5	7,254	6,987	28.45	27.48
Frozen	511.7	493.2	7,096	6,874	27.96	27.04

Table 7. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 3)

Steroid isolated and derivative formed		Specific activity (disintegrations/min/nmole)					
		Normal incubation medium			NAD only under N_2		
		10,000:1	5,000:1	1,000:1	10,000:1	5,000:1	1,000:1
DHA							
	DHA	36.02	32.11	163.4	1,008	1,459	1,779
	DHAAc	34.62	31.02	161.3	991.6	1,408	1,702
DHAS							
	DHA	2,029	2,163	1,979	827.0	535.9	147.1
	DHAAc	2,105	2,127	1,960	810.6	559.7	141.6
Δ^4A							
	Δ^4A	19.15	19.50	23.01	98.01	83.19	86.27
	T	18.47	19.36	22.98	95.42	82.75	83.56

Table 8. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 4)

Steroid isolated and derivative formed	Specific activity (disintegrations/min/nmole)		
	Preincubated		Normal
	+ATP	-ATP	
DHA			
DHA	26.12	937.0	23.04
DHAAc	27.12	979.3	23.48
DHAS			
DHA	2,587	628.7	1,298
DHAAc	2,418	677.3	1,201
Δ^4A			
Δ^4A	37.12	95.82	26.91
T	35.05	91.77	25.80

Table 9. The identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 5)

Steroid isolated and derivative formed	Specific activity (disintegrations/min/nmole)			
	Adult zone	Foetal zone	Whole Tissue	Control
DHA				
DHA	200.7	153.6	1,908	250.1
DHAAc	189.3	142.1	1,840	247.3
A'diol	186.9	138.7	1,734	239.7
DHAS				
DHA	26.34	86.22	1,117	0
DHAAc	24.74	84.08	1,076	0
A'diol	23.98	80.69	1,005	0
Δ^4A				
Δ^4A	0.9176	0.5130	13.80	0
T	0.9413	0.5336	12.87	0
TAc	0.9503	0.5010	13.02	0

Table 16. Incubation of [illegible] DHA with human foetal adrenal tissue (Experiment 6)

Distance from gland surface (µm)	Specific activity (disintegrations/min/nmole)						
	DHA		DHAS		Δ^4A		
	DHA	DHAAc	DHA	DHAAc	Δ^4A	T	TAc
16 - 64	691.2	667.3	0	0	3.987	3.945	3.722
80 - 128	725.8	711.0	0	0	5.602	5.420	5.162
144-192	765.6	743.8	0	0	5.511	5.213	5.112
208-256	541.7	519.8	251.3	238.8	2.983	2.901	2.728
272-320	369.9	375.1	492.4	484.1	3.981	3.803	3.724
336-384	312.5	300.8	664.2	634.9	3.521	3.401	3.238
400-448	296.2	309.0	652.8	635.6	2.601	2.545	2.497
464-512	*	*	279.2	272.4	*	*	*
528-574	568.8	552.2	968.9	930.8	2.479	2.447	2.402
Whole tissue	27,610	27,150	30,390	29,350	238.6	229.3	215.4
Blank	804.3	797.1	0	0	0	0	0

* not investigated

Table 11. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 7)

Distance from gland surface (μm)	Specific activity (disintegrations/min/nmole)					
	DHA		DHAS		Δ^4A	
	DHA	DHAAc	DHA	DHAAc	Δ^4A	T
16 - 64	1,706	1,696	42.99	41.46	38.68	38.35
80 - 128	1,887	1,796	310.8	301.4	12.38	12.34
144 - 192	1,270	1,267	997.2	984.5	7.216	7.030
208 - 256	590.5	588.8	2,101	2,051	7.006	6.806
272 - 320	774.8	804.3	1,825	1,775	6.790	6.727
336 - 384	713.2	726.1	1,873	1,811	8.513	8.293
400 - 448	937.0	897.1	1,869	1,845	7.714	7.550
464 - 512	705.8	730.0	2,043	1,987	4.710	4.500
528 - 576	714.9	706.2	2,032	2,004	5.522	5.257
592 - 640	730.7	710.4	2,071	2,021	3.428	3.562
656 - 704	971.2	928.6	1,860	1,853	3.370	3.500
720 - 768	729.5	728.0	2,059	2,057	6.773	6.412
784 - 832	726.1	720.3	2,485	2,414	5.310	5.281
Whole tissue 5,000:1	1,900	1,979	4,953	4,789	93.01	89.97
Whole tissue 10,000:1	1,245	1,209	6,464	6,352	65.07	64.10
Blank	2,292	2,163	0	0	0	0

Table 12. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 8)

Distance from gland surface (μm)	Specific activity (disintegrations/min/nmole)					
	DHA		DHAS		Δ^4A	
	DHA	DHAAc	DHA	DHAAc	Δ^4A	T
16 - 64	644.1	631.8	16.78	17.42	27.54	27.08
80 - 128	697.3	664.1	17.41	17.78	49.73	47.65
144 - 192	736.8	711.9	20.68	21.31	33.83	32.27
208 - 256	698.2	663.7	36.15	37.36	26.64	26.44
272 - 320	113.6	109.2	1,079	1,122	14.22	13.78
336 - 384	17.89	17.50	1,352	1,365	7.338	7.349
400 - 448	48.85	46.43	1,181	1,134	6.579	6.276
464 - 512	675.2	644.1	46.30	44.58	21.68	20.89
528 - 576	664.7	659.7	112.9	108.7	19.81	19.43
592 - 640	22.09	20.99	1,274	1,334	6.076	5.909
656 - 704	33.58	33.26	1,258	1,271	8.382	8.007
720 - 768	37.46	35.10	1,201	1,242	5.556	5.394
784 - 832	8.523	8.098	1,317	1,356	3.968	3.913
848 - 896	109.8	109.2	1,026	1,071	5.289	5.032
912 - 960	48.03	45.78	1,209	1,271	3.464	3.315
976 - 1,024	114.7	113.2	1,094	1,041	2.958	2.887
Whole tissue	59.22	56.35	3,732	3,889	10.84	10.31
Blank	891.2	875.1	0	0	0	0

Table 13. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 9)

Distance from gland surface (μm)	Specific activity (disintegrations/min/nmole)					
	DHA		DHAS		Δ^4A	
	DHA	DHAAc	DHA	DHAAc	Δ^4A	T
16 - 64	960.2	912.5	2.345	2.290	20.21	19.34
80 - 128	932.1	899.7	1.478	1.346	24.60	23.59
144 - 192	908.3	896.1	2.901	3.056	32.01	31.54
208 - 256	865.2	835.4	3.419	3.000	31.83	30.95
272 - 320	851.2	844.5	5.402	5.360	24.58	24.31
336 - 384	819.3	800.5	4.012	3.469	25.58	25.02
400 - 448	834.5	799.3	30.98	28.14	38.84	38.71
464 - 512	782.0	754.2	24.13	27.02	73.62	72.34
528 - 576	833.5	831.4	31.70	31.37	30.48	29.94
592 - 640	828.7	791.7	70.15	67.75	31.10	29.63
656 - 704	861.3	835.2	13.68	13.91	28.30	29.01
720 - 768	851.2	810.1	30.82	28.56	43.24	42.29
784 - 832	845.7	790.0	33.17	36.21	40.92	39.02
848 - 896	841.3	793.3	28.32	28.56	37.14	38.21
912 - 960	830.4	804.6	23.87	22.25	37.08	36.98
Whole tissue +ATP	7,615	7,218	8,415	7,514	251.5	247.8
Whole tissue -ATP	13,700	13,480	333.2	327.4	897.9	869.1
Blank	998.6	960.4	0	0	0	0

Table 14. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 10)

Distance from gland surface (μm)	Specific activity (disintegrations/min/nmole)					
	DHA		DHAS		Δ^4A	
	DHA	DHAAc	DHA	DHAAc	Δ^4A	T
16 - 64	2,579	2,503	2.869	2.738	18.80	18.01
80 -128	2,530	2,498	4.094	3.915	23.88	24.20
144 -192	2,415	2,318	6.931	6.744	67.31	65.18
208 -256	2,132	2,035	7.301	7.102	235.4	228.1
272 -320	2,084	1,993	7.550	7.460	215.0	212.8
336 -384	2,344	2,315	48.11	46.50	46.28	45.06
400 -448	2,338	2,294	95.31	92.18	27.26	26.77
464 -512	2,340	2,311	80.05	79.23	28.84	27.59
528 -576	2,300	2,187	85.38	83.01	33.01	33.11
592 -640	2,268	2,237	126.2	121.5	35.96	34.31
656 -704	2,287	2,190	130.5	129.8	38.24	39.04
720 -768	2,263	2,206	135.2	133.4	50.31	48.93
784 -832	2,304	2,283	143.2	139.7	38.64	37.88
848 -896	2,185	2,099	175.3	174.8	36.61	36.09
912 -960	2,289	2,274	110.1	106.3	34.58	33.61
976 -1,024	2,286	2,234	131.4	127.2	34.37	33.98
Whole tissue	4,581	4,713	369.1	361.8	179.1	175.4
Blank	2,596	2,491	0	0	0	0

Table 15. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with adrenal tissue from a newborn female hydrocephalic infant (Experiment 11)

Steroid isolated and derivative formed	Specific activity (disintegrations/min/nmole)				
	Adult zone	Foetal zone	Whole Tissue		Control
			10,000:1	100,000:1	
DHA					
DHA	48.14	94.20	41.89	2,479	110.1
DHAAc	46.34	96.41	41.32	2,304	113.4
A'diol	43.98	97.91	40.21	2,450	119.6
DHAS					
DHA	4.448	35.02	542.7	15,336	0
DHAAc	4.387	31.84	535.2	16,078	0
A'diol	4.352	33.74	520.9	14,563	0
Δ^4A					
Δ^4A	31.09	5.171	216.9	2,677	3.713
T	32.33	5.389	197.9	2,831	3.509
TAc	30.49	5.524	203.2	2,745	3.740
11βΔ^4A					
11βΔ^4A	8.172	3.753	14.29	57.69	2.654
11βT	8.666	3.814	15.01	52.34	2.706
11βTAc	8.032	3.892	15.32	54.29	2.549

Table 16. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with adrenal tissue from a newborn female anencephalic infant of 42 weeks gestation. (Experiment 12)

Distance from gland surface (μm)		Specific activity (disintegrations/min/nmole)								
		DHA		DHAS		Δ^4A		11βΔ^4A		
		DHA	DHAAc	DHA	DHAAc	Δ^4A	T	11βΔ^4A	11βT	11βTAc
16 - 64		594.0	569.1	19.11	18.68	518.9	497.4	9.021	9.215	8.811
80 - 128	*	389.8	377.1	17.75	18.01	733.5	703.8	10.46	10.18	10.20
144 - 192		175.2	170.2	720.4	752.7	538.0	525.4	11.53	11.48	11.36
400 - 448	*	644.7	614.5	57.79	58.46	472.9	457.2	17.78	17.57	17.46
528 - 576		139.7	134.5	733.6	757.1	628.0	614.5	30.38	30.51	29.98
656 - 704	*	678.7	648.1	39.11	41.02	340.3	325.6	13.81	13.97	13.81
784 - 832		154.5	153.2	1,012	983.5	390.0	380.9	5.764	5.606	5.625
912 - 960	*	600.8	597.2	45.02	45.90	393.0	392.2	8.379	8.151	7.973
1040-1088		188.0	185.7	1,273	1,233	298.5	283.6	9.715	9.530	9.550
1168-1216	*	621.7	618.9	58.28	58.13	277.8	274.4	15.95	15.36	15.19
Whole Tissue		2,118	2,025	967.3	927.0	2,598	2,502	80.72	80.26	80.11
Whole Tissue*		2,025	1,949	76.15	74.99	3,324	3,202	180.1	179.5	177.5
Blank		1,103	1,067	0	0	0	0	0	0	0

* incubated in absence of added ATP

Table 17. Identification of steroids isolated from the incubation of [7α-^{3}H] DHA with adrenal tissue from a newborn anencephalic infant of 38 weeks gestation (Experiment 13)

Distance from gland surface (μm)	Specific activity (disintegrations/min/nmole)							
	DHA		DHAS		Δ^4A		11β Δ^4A	
	DHA	DHAAc	DHA	DHAAc	Δ^4A	T	11β Δ^4A	11T
16 - 64	1,445	1,402	10.62	10.32	20.73	19.77	1.175	1.136
144 - 192	1,127	1,072	193.1	188.1	63.15	62.82	3.457	3.298
272 - 320	562.6	536.0	838.9	798.3	79.14	76.67	2.652	2.537
400 - 448	150.8	147.2	1,272	1,216	30.86	29.42	2.421	2.308
16 - 64	1,209	1,172	182.6	178.0	43.19	41.08	1.565	1.536
144 - 192	421.1	404.7	809.0	803.4	67.16	64.89	1.646	1.572
Whole tissue +ATP	1,568	1,553	5,056	4927	301.5	297.2	12.12	11.98
Whole Tissue -ATP	5,437	5,219	632.9	627.3	590.7	569.8	20.34	19.52
Blank	1,496	1,433	0	0	0	0	0	0

Table 18. Further identification of steroids isolated from the incubation of [7α-^{3}H] DHA with human foetal adrenal tissue (Experiment 6)

Steroid	Source	Specific activity (disintegrations/min/mg)			
		Crystals		Mother liquors	
		I	II	I	II
DHA (as DHAAc)	A *	46,640	45,720	48,100	46,980
	B +	440,800	448,800	459,300	451,400
DHAS (as DHAAc)	A	15,130	14,990	15,230	15,210
	B	315,800	307,100	319,400	313,500
Δ^4A (as TAo)	A	242.0	240.5	251.3	245.0
	B	1,963	1,942	1,987	1,968

* Combined extracts from the incubations with tissue sections.

\+ Extract from whole tissue incubation.

Table 19. Further identification of steroids isolated from the incubation of [7α-^{3}H] DHA with adrenal tissue from a newborn anencephalic infant of 42 weeks gestation (Experiment 12)

Steroid	Source *	Specific activity (disintegrations/min/mg)			
		Crystals		Mother Liquors	
		I	II	I	II
DHA (as DHAAc)	A	15,550	15,330	15,700	15,440
	B	14,020	13,820	14,170	13,920
	C	5,437	5,192	5,416	5,309
	D	11,310	11,030	11,430	11,130
DHAS (as DHAAc)	A	4,316	4,258	4,399	4,286
	B	410.5	404.1	420.1	408.7
	C	14,510	14,420	14,740	14,715
	D	321.7	329.2	335.7	320.0
Δ^4A (as Δ^4A)	A	21,420	21,960	21,560	21,600
	B	25,310	26,090	25,160	26,395
	C	15,060	14,840	15,520	15,220
	D	21,980	21,600	21,310	21,810

* A Homogenate with ATP
B Homogenate without ATP
C Combined tissue section incubations with ATP
D Combined tissue section incubations without ATP

(expt. 1; Fig. 4) converted 50% of the incubated [^{3}H] DHA to its sulphate within 5 min. The rate of sulphoconjugation reduced thereafter, attaining a plateau after 15 min. Formation of androstenedione increased throughout the 30 min period studied.

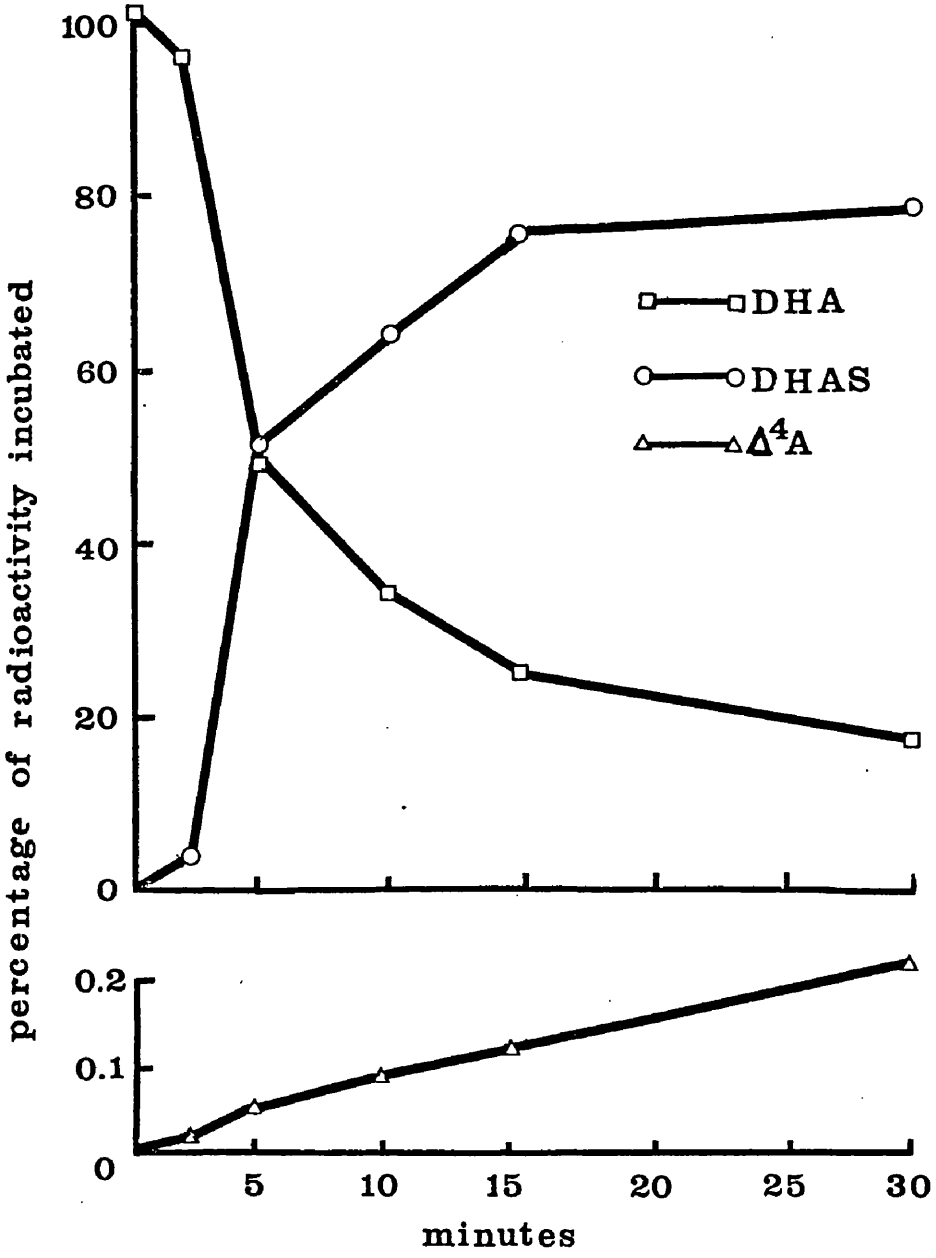
The rate of DHA sulphate formation by tissue from a foetus of 17 weeks gestation (expt.2; Fig. 5) did not decrease appreciably until 30 min of incubation, when more than 90% of the [^{3}H] DHA had been metabolized. Androstenedione production increased linearly during the 2 h incubation period.

Conversion of [^{3}H] DHA to DHA sulphate was, therefore, under the in vitro conditions employed, more rapid than to androstenedione.

The effect of tissue:steroid ratio on [^{3}H] DHA metabolism.

Adrenal tissue from a foetus of 24 weeks gestation (expt. 7) metabolized less [^{3}H] DHA to its sulphate (51.80 & 68.14%) and slightly more to androstenedione (1.44 & 1.02%) at tissue:steroid 5,000:1 than 10,000:1. Less marked differences between the patterns of DHA metabolism at these tissue: steroid ratios were apparent in experiment 3 (Fig. 6). This tissue effected formation of less DHA sulphate and more

Figure 4. Formation of DHAS and Δ^4A from DHA by the adrenal gland of a previable human foetus as a function of time (Expt. 1).



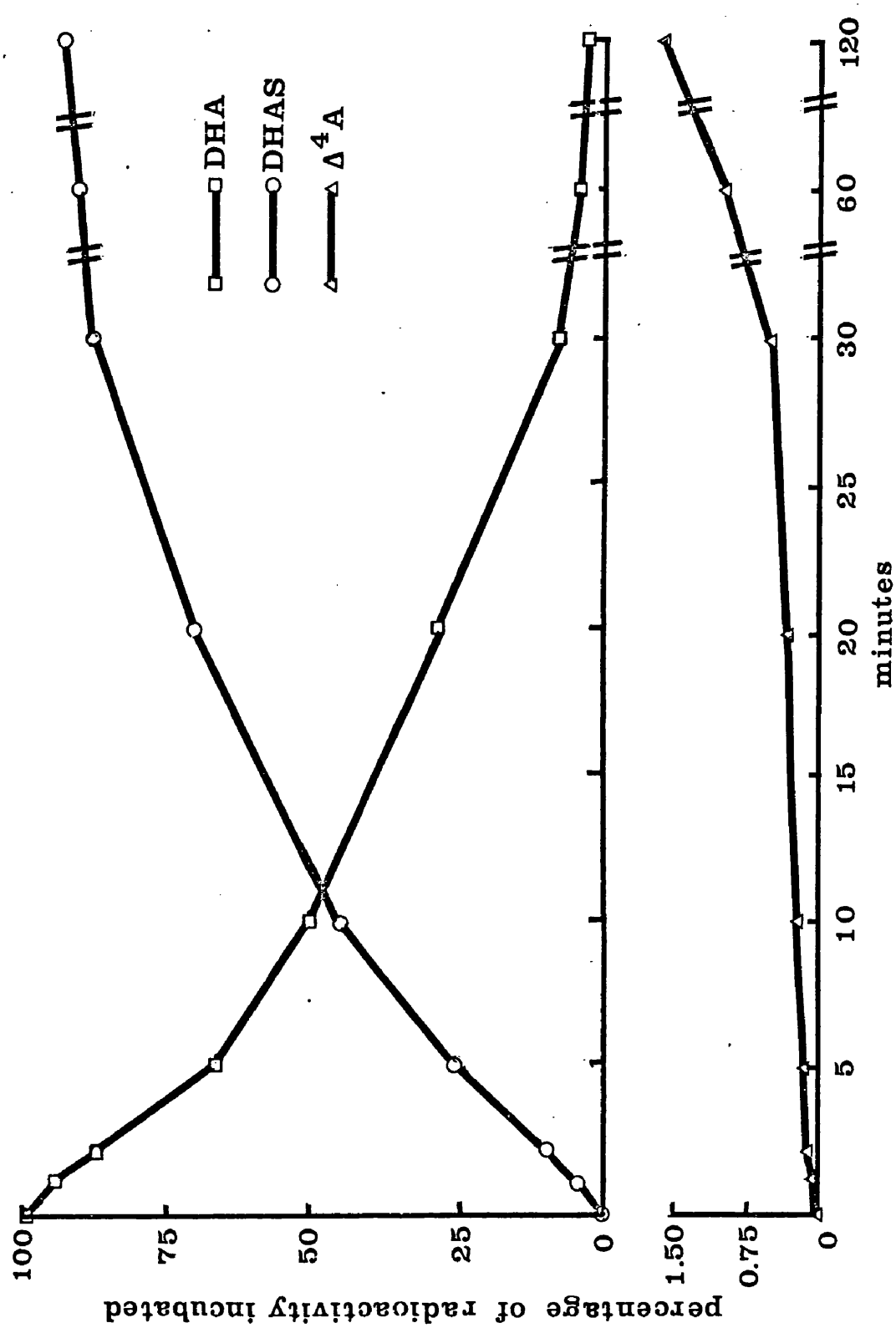
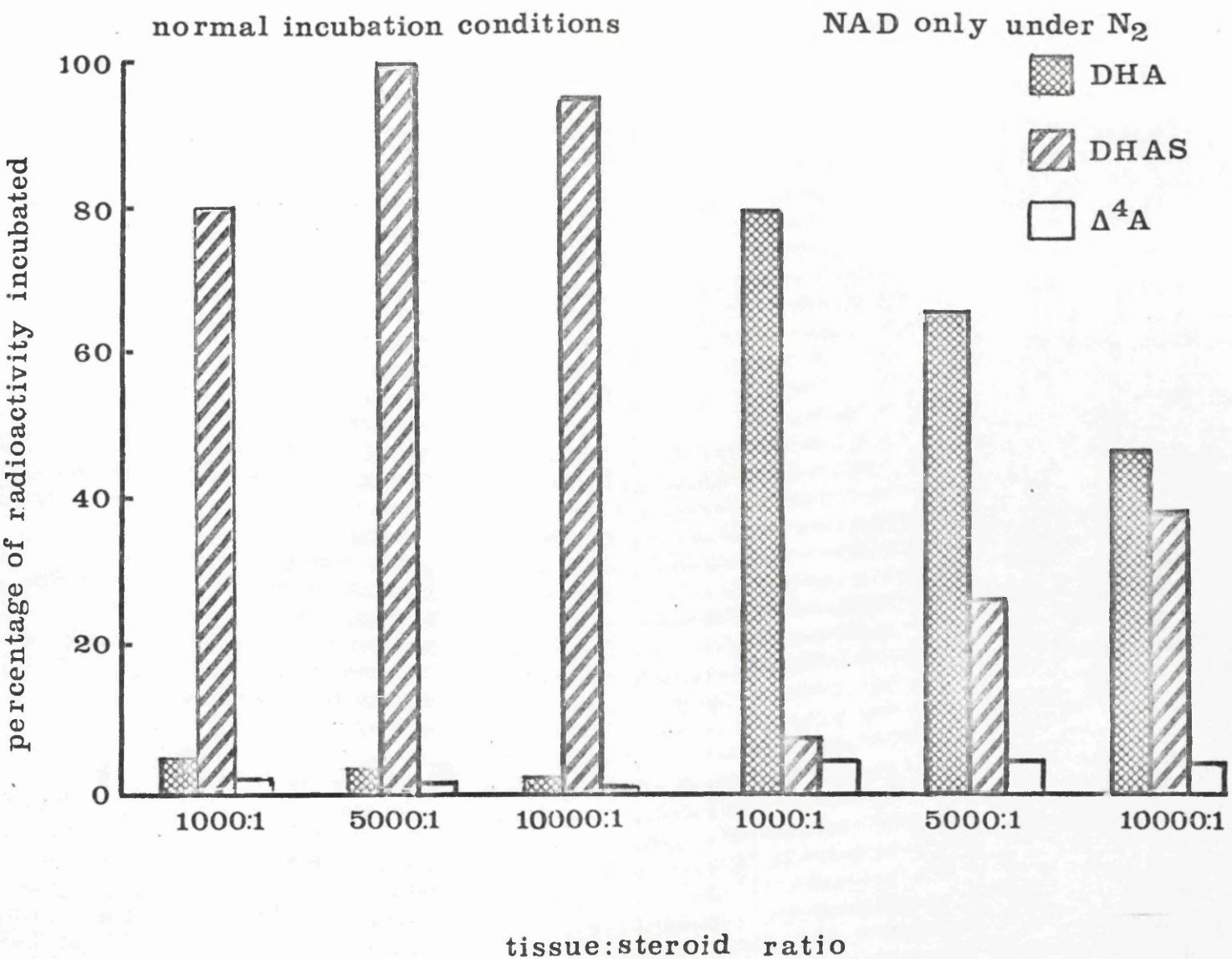


Figure 5. Formation of DHAS and Δ^4A from DHA by the adrenal gland of a previable human foetus as a function of time (Expt. 2).

Figure 6. Effect of tissue:steroid ratio and incubation under N_2 with NAD as only added co-factor on DHA metabolism by the adrenal gland of a previable human foetus (Expt.3).



androstenedione from [^{3}H] DHA at tissue:steroid 1,000:1 than at the higher ratios (1,000:1, 81 & 1.3%; 5,000:1, 99 & 0.9%; 10,000:1, 96 & 0.9%, respectively). DHA metabolism by adrenal tissue from a foetus of 17 weeks gestation (expt. 2; Fig. 7) incubated at tissue:steroid 500:1 was much less than that encountered at higher ratios. Variation in the amounts of DHA sulphate formed was much greater between tissue: steroid 500:1 and 2,000:1 (8.8 - 70.5%) than between ratios of 5,000 - 100,000:1 (81.7 - 93.6%). Androstenedione formation declined markedly between tissue:steroid 500:1 and 1,000:1 and reduced gradually thereafter as the ratios increased.

Higher tissue:steroid ratios, therefore favoured sulpho-conjugation of [^{3}H] DHA and lower ratios its conversion to androstenedione.

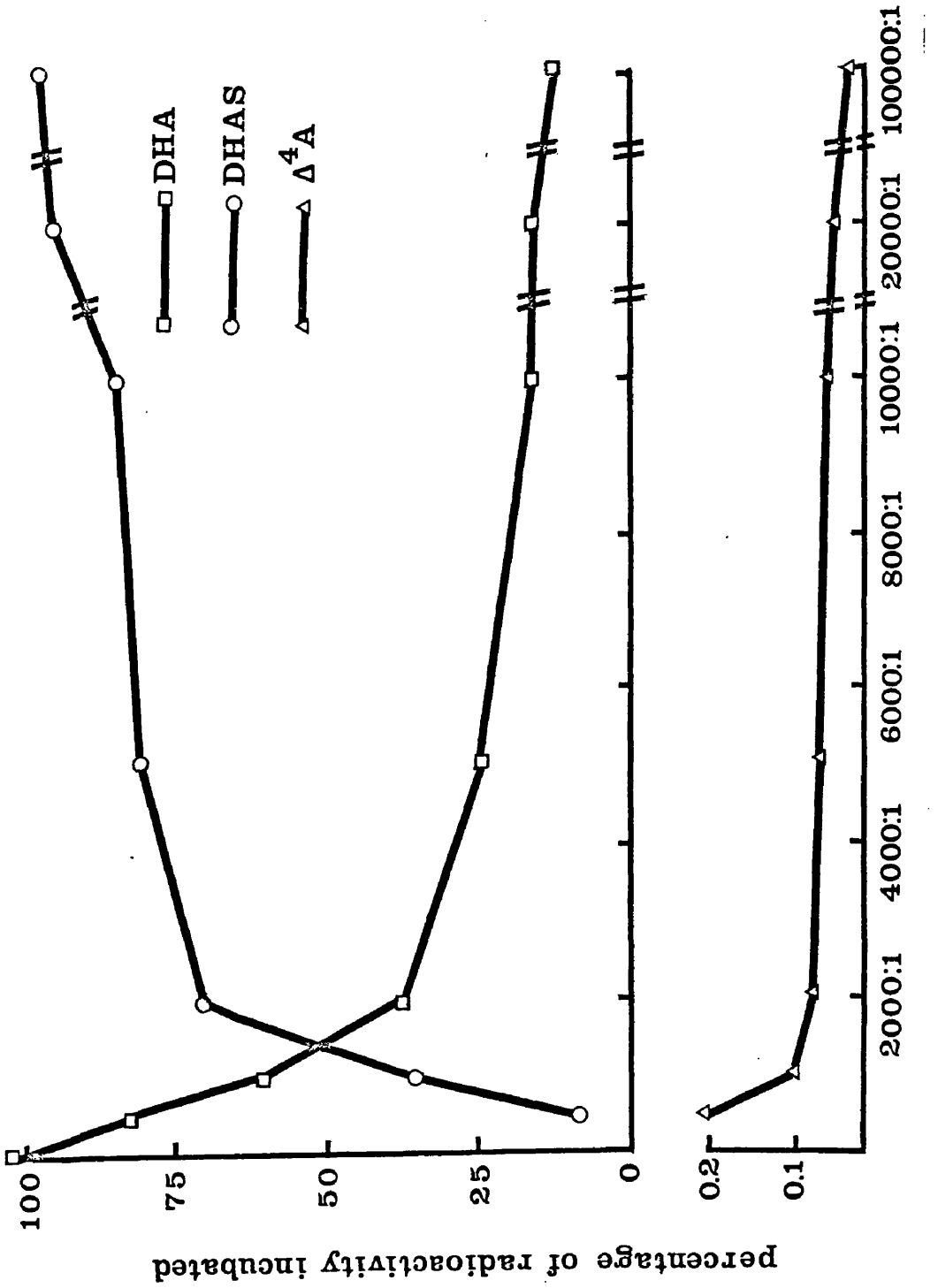
The effect of preincubation of foetal adrenal tissue on [^{3}H] DHA metabolism.

Preincubation of foetal adrenal tissue for 30 min prior to addition of [^{3}H] DHA (expt. 4) had little effect on the formation of DHA sulphate (preincubated, 91.48%; normal, 90.34%) or androstenedione (preincubated, 1.96%; normal 1.83%).

The effect of storage of tissue on [^{3}H] DHA metabolism.

Conversions of [^{3}H] DHA to DHA sulphate and

Figure 7. Effect of tissue:steroid ratio on DHA metabolism by the adrenal gland of a previable human foetus (Expt. 2).



androstenedione were unaltered by previous freezing of the tissue in solid CO_2 or storage of the homogenate in ice (expt. 2; Table 20).

The effect of the omission of ATP from the incubation medium on [^{3}H] DHA metabolism.

Omission of ATP as an added co-factor in the incubation medium resulted in markedly decreased conversions of [^{3}H] DHA to its sulphate by adrenal glands from previable human foetuses (expts. 4 & 9; Fig. 8). The increases in androstenedione formation in the absence of ATP did not balance the lower levels of sulphoconjugation, which were reflected by a decrease in the amounts of DHA metabolized.

Similar results were obtained (see below) with adrenal tissue from newborn anencephalic infants in the presence and absence of exogenous ATP, indicating a requirement of this co-factor for sulphoconjugation of DHA *in vitro* by the adrenal glands of the human foetus and newborn infant.

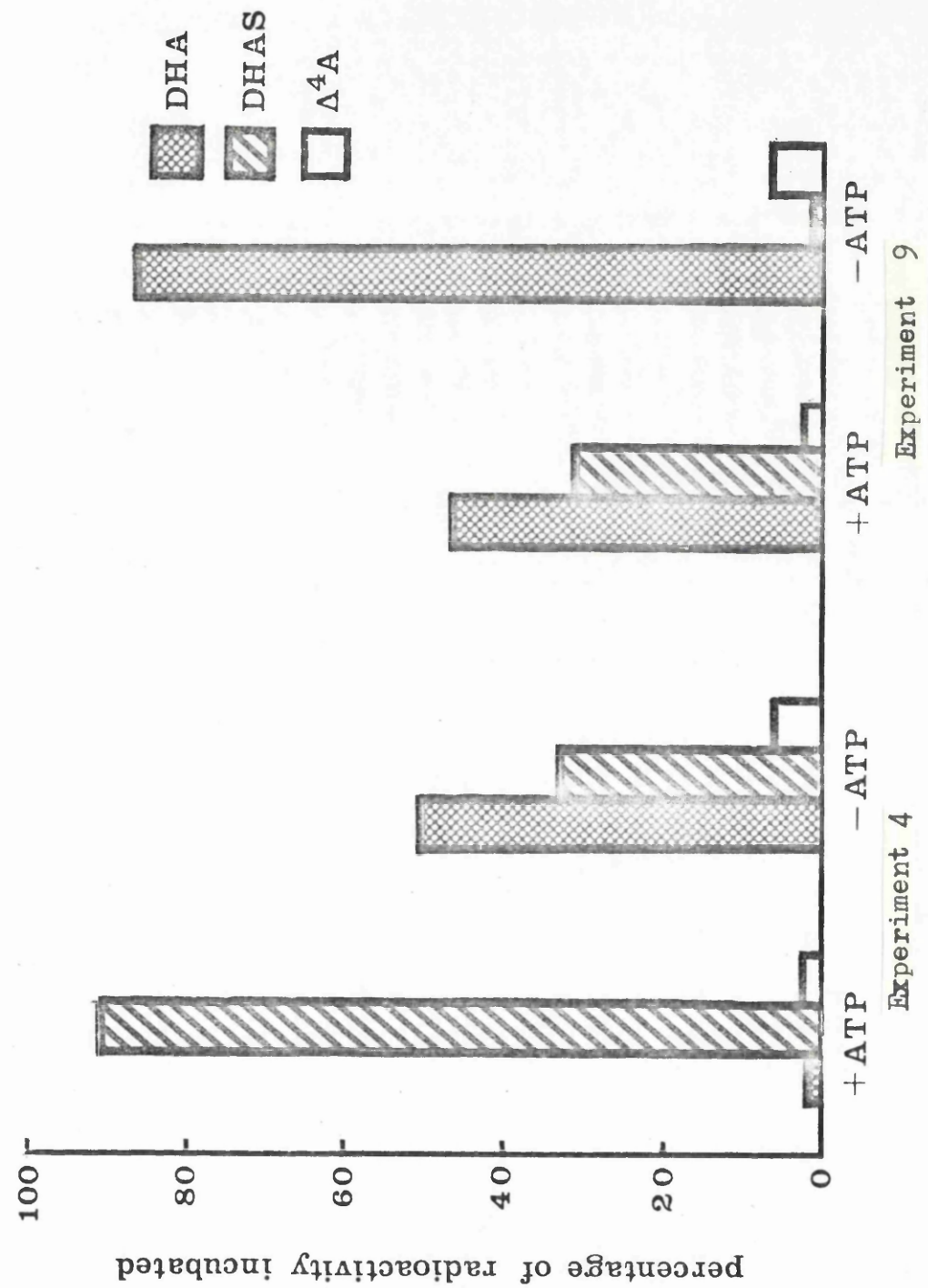
The effect of incubation of foetal adrenal tissue under nitrogen with NAD as the only added co-factor on [^{3}H] DHA metabolism.

The patterns of [^{3}H] DHA metabolism to DHA sulphate

Table 20. The metabolism of [7α-^{3}H] DHA by human foetal adrenal tissue following storage in ice or freezing in solid CO_2 (Experiment 2)

Tissue	Percentage of radioactivity incubated present in isolated steroid		
	DHA	DHAS	Δ^4A
Fresh	8.59	88.09	0.52
Frozen in solid CO_2 for 2 h.	8.86	83.29	0.49
Homogenate stored in ice for 1½ h.	8.64	84.91	0.50

Figure 8. Effect of omission of ATP from the incubation medium on DHA metabolism by adrenal glands of previable human foetuses.



and androstenedione by foetal adrenal tissue incubated under nitrogen in medium containing NAD as the only added co-factor at the three tissue:steroid ratios investigated (Fig. 6) were similar to those observed in experiments conducted in the absence of exogenous ATP i.e. reduced formation of DHA sulphate and increased conversion to androstenedione.

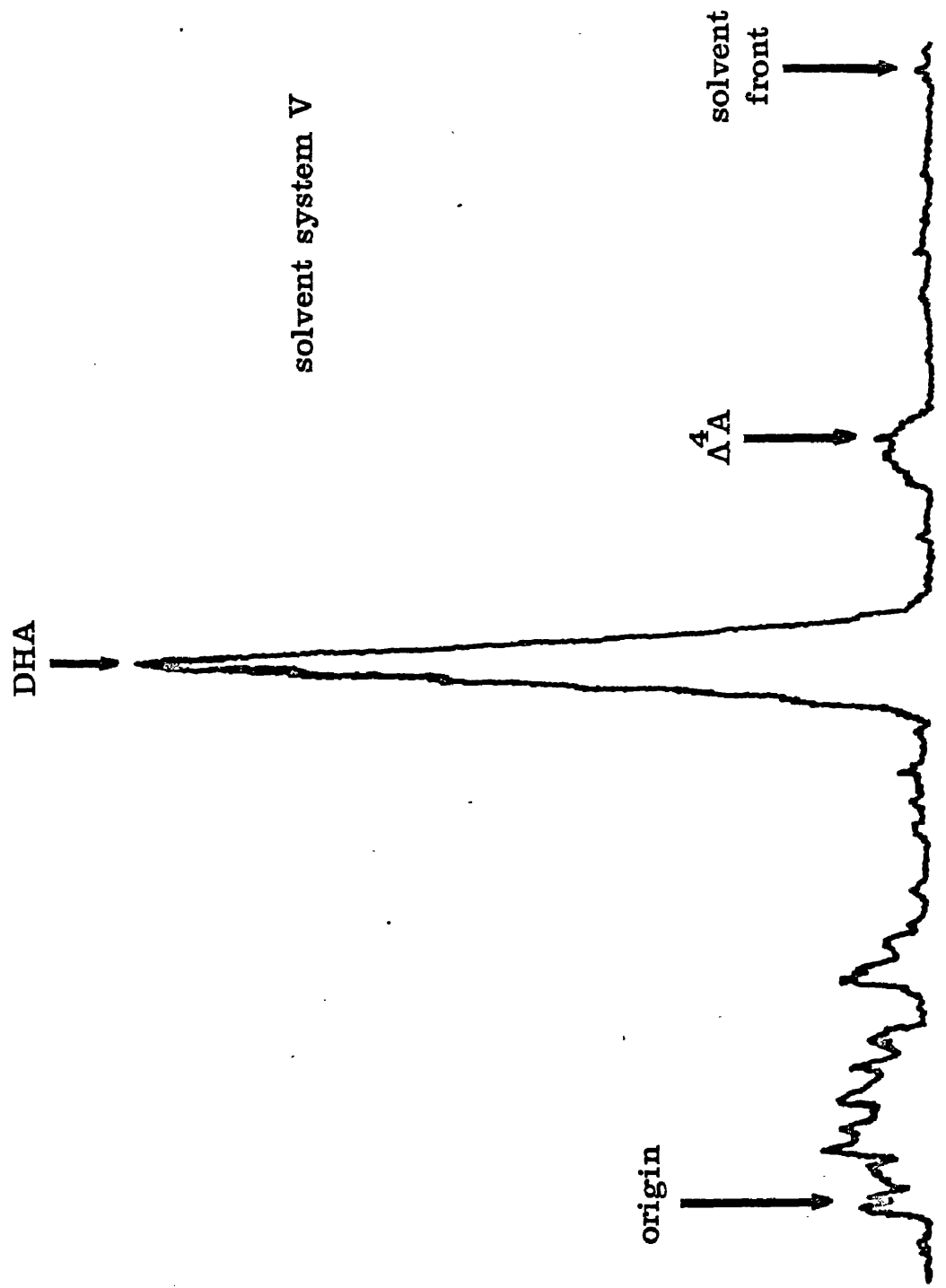
Metabolism of [^{3}H] DHA by whole tissue preparations of adrenal glands from previable human foetuses.

Conversions of [^{3}H] DHA to DHA sulphate and androstenedione by homogenized or chopped adrenal glands obtained from human foetuses of 13 - 24 weeks gestation are presented in Table 21. Incubations were for 30 min at tissue:steroid 10,000:1 in the presence of exogenous ATP. No radioactivity could be detected in association with carrier 11β-hydroxyandrostenedione in experiment 6. This steroid was not investigated in the other incubations with adrenal tissue from previable human foetuses. Radiochromatogram scanning of the benzene-chloroform extracts (e.g. Fig. 9) invariably revealed the presence of radioactivity in compounds more polar than DHA. None of

Table 21. Metabolism of [7α-^{3}H] DHA by adrenal glands from previable human foetuses incubated at tissue:steroid 10,000:1 for 30 min.

Experiment	Tissue Preparation	Gestational age (weeks)	Conversion (%)		
			DHA	DHAS	Δ^4A
1	Homogenate	15	1.13	74.40	0.21
2	"	17	8.72	88.09	0.52
3	"	15	1.63	95.53	0.88
4	Chopped	13	2.49	90.34	2.83
5	"	22	58.23	34.52	0.42
6	"	18	42.03	31.03	0.35
7	Homogenate	24	10.10	68.14	1.02
9	"	15	47.26	31.24	1.60

Figure 9. Radiochromatogram of benzene–chloroform extract of an incubation of $[^3H]_{DHA}$ with the adrenal gland of a previable human foetus.



these "metabolites" appeared to represent more than 1 - 2% of the incubated radioactivity. No evidence was obtained following radiochromatogram scanning for the presence in the solvolysed steroid extracts for any radioactive compound other than DHA.

With the exception of experiments 4 and 9, tissue homogenates metabolized more [^{3}H] DHA than did the chopped glands. Neither the amounts of DHA metabolized nor the conversions to DHA sulphate and androstenedione could be correlated with the gestational age or the sex of the foetuses studied.

In all cases much more [^{3}H] DHA was sulphoconjugated (31 - 95%) than was converted to androstenedione (0.2 - 2.8%). DHA sulphate was the principal metabolite of [^{3}H] DHA formed under the conditions employed.

The metabolism of [^{3}H] DHA by adult and foetal zone tissue from the adrenal gland of a previable human foetus.

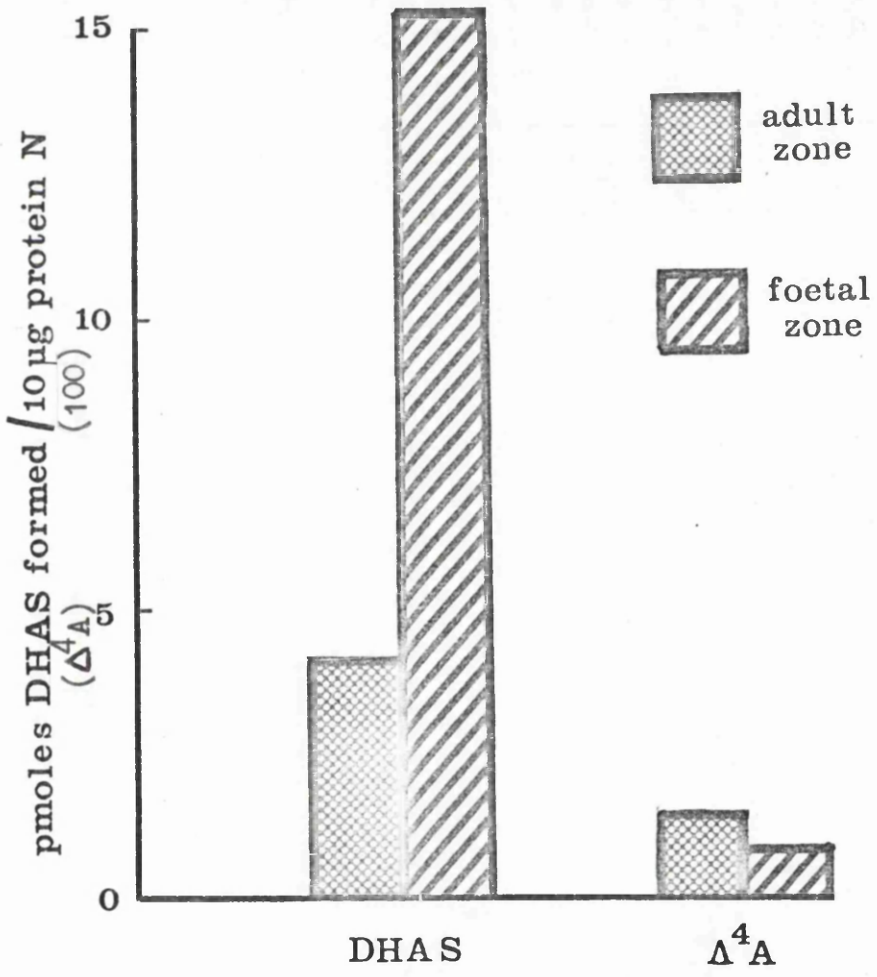
Foetal zone tissue prepared in experiment 5 (Table 22; Fig. 10)* metabolized more [^{3}H] DHA than did adult zone

* The results of experiments with tissue sections are presented as percentages of the incubated radioactivity in Tables 22 - 30 and as pmoles radioactive steroid formed / 10 or 100 µg protein nitrogen in Figs. 10 - 19.

Table 22. Metabolism of [7α-^{3}H] DHA by adrenal tissue preparations from a foetus of 22 weeks gestation (Experiment 5)

Tissue	Percentage of radioactivity incubated present in isolated steroid		
	DHA	DHAS	Δ^{4}A
Adult zone	92.36	7.21	0.27
Foetal zone	69.54	27.53	0.15
Chopped gland	58.23	34.52	0.42
Control	98.33	0	0

Figure 10. DHA metabolism by histologically-defined adrenal tissue from a previable human foetus (Expt. 5).



tissue. Conversions to DHA sulphate were approximately four times greater in the foetal than in the adult zone, whereas the latter zone formed approximately twice as much androstenedione. Conversions to the latter steroid (0.15 & 0.27%) were much smaller than to DHA sulphate (27.5 & 7.2%). The metabolism of [^{3}H] DHA by the chopped tissue preparation was consistent with that observed in the separated zones,

The quantitative histological distribution of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities in the adrenal glands of previable human foetuses.

a. In the presence of exogenous ATP.

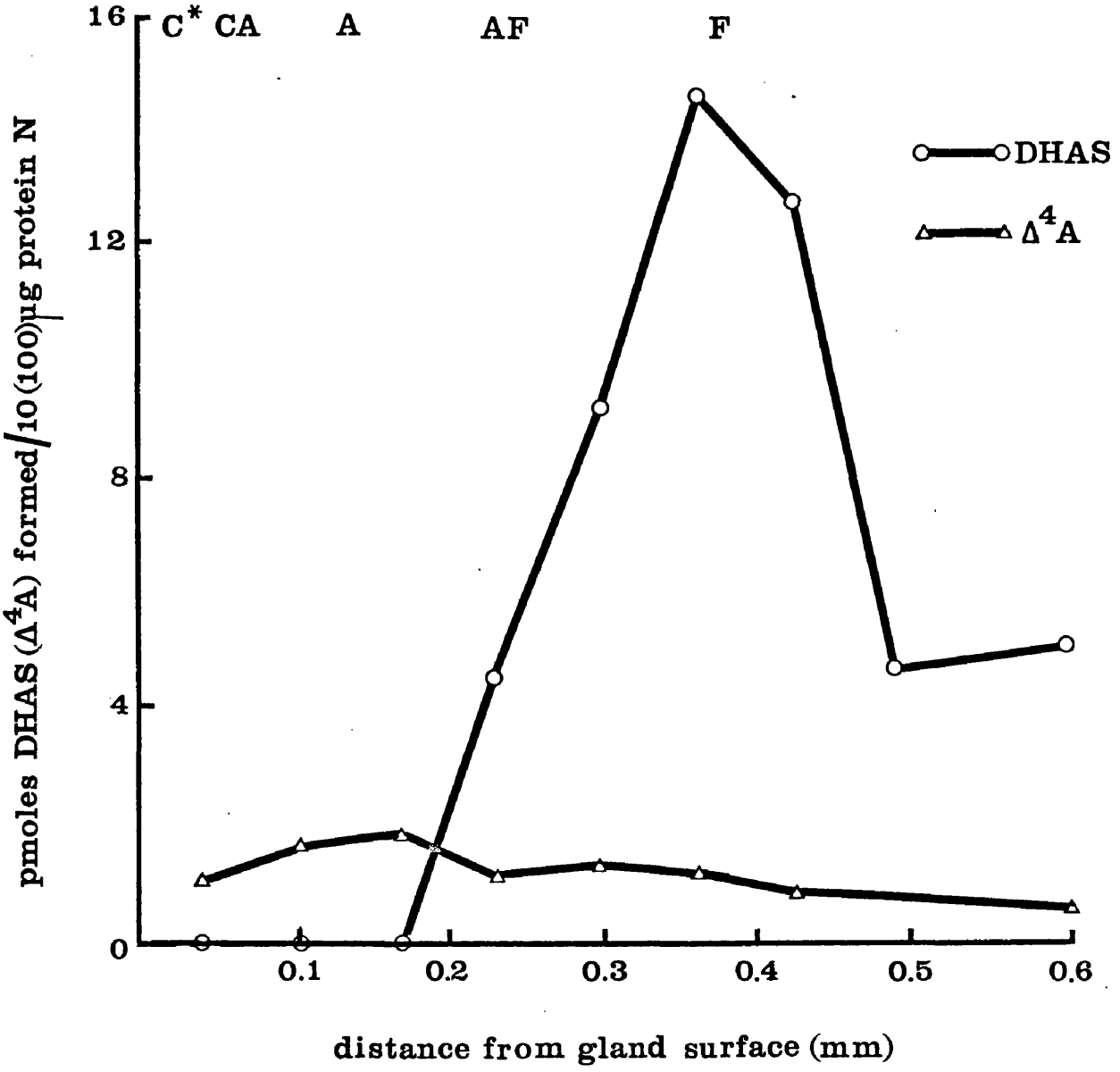
Experiment 6. (Table 23; Fig. 11) No conversion of [^{3}H] DHA to its sulphate was detected at the surface of the gland. The formation of this steroid increased sharply at the adult/foetal zone junction to attain a peak at the periphery of the foetal zone. Conversions to androstenedione (maximum 0.7%) were much lower than to DHA sulphate (maximum 54%) and were slightly greater in the adult (around 0.7%) than in the foetal zone (around 0.3%).

Table 23. Metabolism of [7α-^{3}H] DHA by adrenal tissue from a foetus of 18 weeks gestation incubated at tissue:steroid 10,000:1 (Experiment 6)

Distance from gland surface (μm)	Percentage of radioactivity incubated present in isolated steroid		
	DHA	DHAS	Δ^4A
16 - 64	83.44	0	0.48
80 - 128	87.11	0	0.67
144 - 192	92.69	0	0.66
208 - 256	65.19	19.95	0.35
272 - 320	45.75	39.88	0.47
336 - 384	37.67	53.42	0.42
400 - 448	37.16	53.51	0.32
464 - 512	*	22.92	*
528 - 704	68.83	26.31	0.30
Whole tissue	42.03	31.03	0.35
Control	98.34	0	0

* not investigated

Figure 11. Distribution of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities in the adrenal gland of a previable human foetus (Expt. 6).



* C capsule A adult zone F foetal zone
combined letters denote zone junctions

Experiment 7. (Table 24; Fig. 12) DHA sulphate formation again rose sharply as the proportion of foetal zone cells in the tissue sections increased. There was, however, no distinct peak of DHA sulphokinase activity. Conversions to androstenedione (maximum 1.7%) were again much lower than to DHA sulphate (maximum 73%) and decreased with increased depth in the gland.

Experiment 8. (Table 25; Fig. 13) DHA sulphokinase activity was low at the surface of the gland and increased at the adult/foetal zone junction to form a peak at the edge of the foetal zone. DHA sulphate formation decreased thereafter to levels only slightly higher than those observed in the adult zone and then increased to attain a second peak deeper in the foetal zone. Conversions to androstenedione increased from the surface of the gland to the adult zone and then declined as the foetal zone was entered. A slight peak of 3β-hydroxysteroid dehydrogenase-isomerase activity was observed in the foetal zone between the peaks of DHA sulphate formation. Conversions to DHA sulphate (maximum 99%) were much greater than to androstenedione (maximum 3%).

These results are consistent with those obtained

Table 24. Metabolism of [7α-^{3}H] DHA by adrenal tissue from a foetus of 24 weeks gestation incubated at tissue:steroid 5,000:1 (Experiment 7)

Distance from gland surface (μm)	Percentage of radioactivity incubated present in isolated steroid		
	DHA	DHAS	Δ^4A
16 - 64	75.38	1.27	1.72
80 - 128	81.61	9.18	0.55
144 - 192	56.26	29.71	0.32
208 - 256	26.14	62.64	0.31
272 - 320	34.98	53.97	0.30
336 - 384	31.91	55.22	0.38
400 - 448	40.66	55.67	0.34
464 - 512	31.73	60.41	0.21
528 - 576	31.48	60.50	0.29
592 - 640	31.94	61.34	0.16
656 - 704	42.03	55.67	0.15
720 - 768	32.18	61.89	0.29
784 - 832	32.10	73.45	0.24
Whole tissue 10,000:1	20.10	68.14	1.02
Whole tissue 5,000:1	30.27	51.80	1.44
Control	98.73	0	0

Figure 12. Distribution of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities in the adrenal gland of a previable human foetus (Expt. 7).

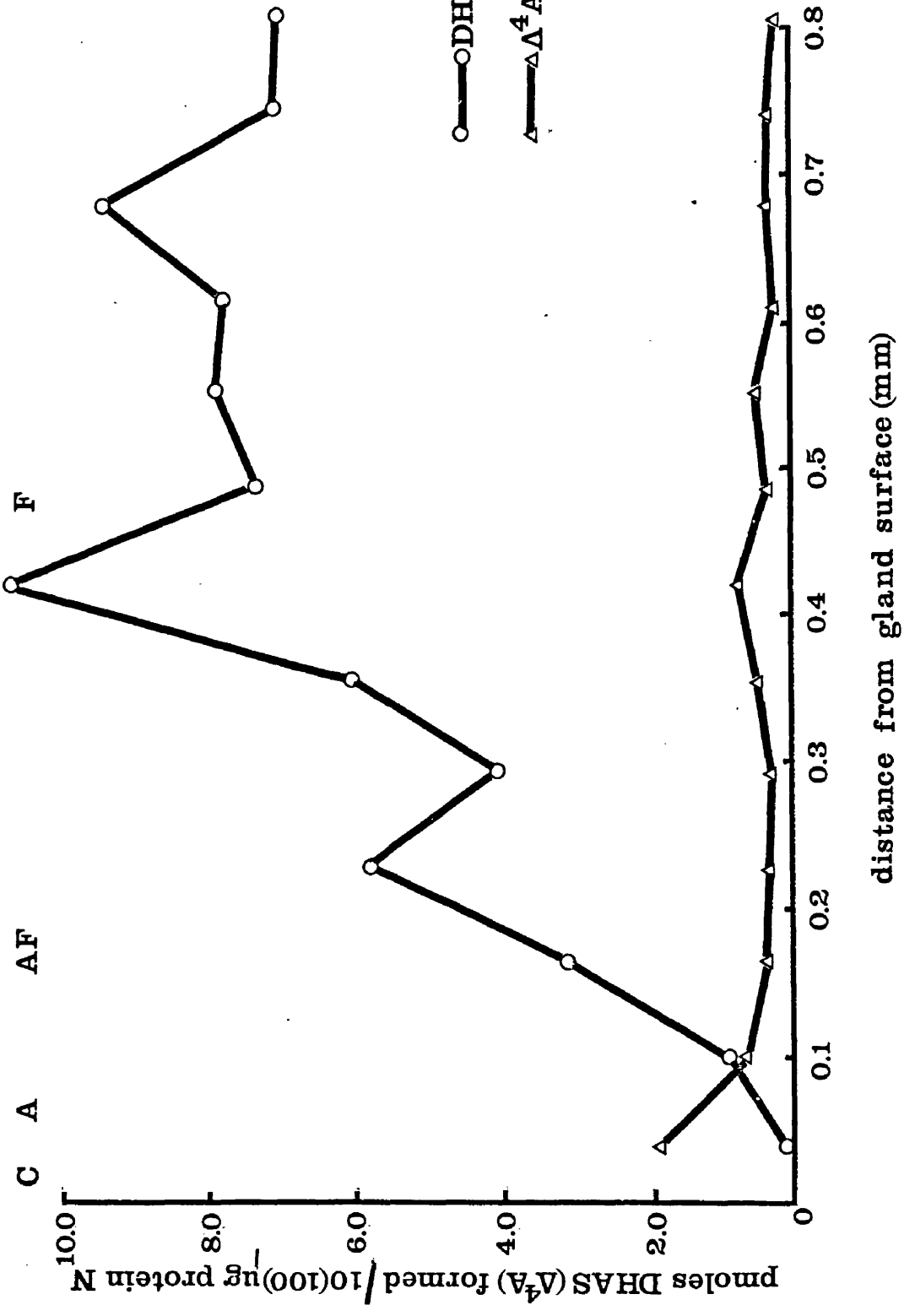


Table 25. Metabolism of [7α-^{3}H] DHA by adrenal tissue from a foetus of 15 weeks gestation incubated at tissue:steroid 10,000:1 (Experiment 8)

Distance from gland surface (μm)	Percentage of radioactivity incubated present in isolated steroid		
	DHA	DHAS	Δ^4A
16 - 64	68.90	1.25	2.97
80 - 128	73.51	1.29	5.29
144 - 192	78.23	1.53	3.59
208 - 256	73.54	2.69	2.83
272 - 320	12.03	80.44	1.52
336 - 384	1.91	99.29	0.80
400 - 448	5.14	84.60	0.70
464 - 512	71.24	3.32	2.32
528 - 576	71.51	8.10	2.13
592 - 640	2.33	95.27	0.65
656 - 704	3.61	92.49	0.89
720 - 768	3.92	89.28	0.60
784 - 832	0.90	97.68	0.43
848 - 896	11.82	76.64	0.56
912 - 960	5.07	90.59	0.37
976 - 1024	12.31	78.03	0.32
Whole tissue	1.13	74.40	0.21
Control	96.07	0	0

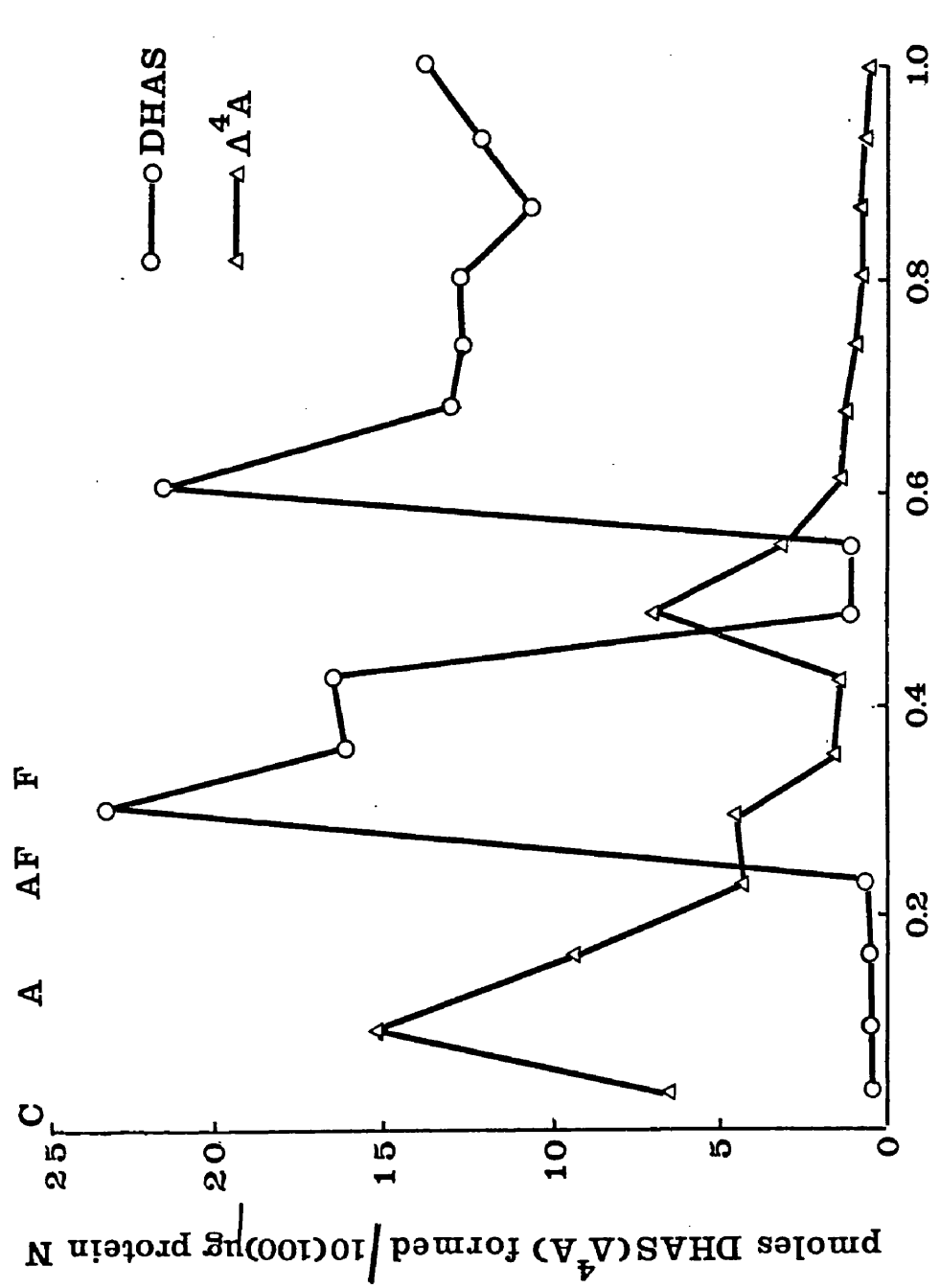


Figure 13. Distribution of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities in the adrenal gland of a previable human foetus (Expt. 8).

in experiment 5, indicating a greater activity of 3β-hydroxy-steroid dehydrogenase-isomerase activity in the adult zone than in the foetal zone and a restriction of DHA sulphokinase activity to, in the main, the foetal zone of the adrenal gland of the previable human foetus. Evidence was obtained in two of the three experiments for the existence near the adult/foetal zone junction of cells which possess greater DHA sulphokinase activity than cells from other parts of the glands.

b. In the absence of exogenous ATP.

Experiment 9. (Table 26; Fig. 14) Conversions of [^{3}H] DHA to its sulphate (maximum 4.4%) were much smaller and to androstenedione greater (maximum 7.6%) than those observed in the presence of added ATP. With the exception of one of the tubes containing foetal zone tissue, more [^{3}H] DHA was converted to androstenedione than to DHA sulphate. Androstenedione formation increased with increased depth in the gland and attained a peak at the adult/foetal zone junction. The conversions of [^{3}H] DHA to androstenedione in the foetal zone (3.0 - 4.5%) were greater than in the adult zone (2.6 - 3.3%).

Table 26. Metabolism of 7α-^{3}H DHA by adrenal tissue from a foetus of 15 weeks gestation incubated at tissue:steroid 10,000:1 in the absence of exogenous ATP (Experiment 9)

Distance from gland surface (μm)	Percentage of radioactivity incubated present in isolated steroid		
	DHA	DHAS	^{4}A
16 - 64	97.32	0.15	2.07
80 - 128	95.19	0.09	2.52
144 - 192	93.76	0.19	3.33
208 - 256	89.41	0.21	3.29
272 - 320	88.12	0.34	2.56
336 - 384	84.17	0.24	2.65
400 - 448	84.90	1.89	4.06
464 - 512	79.93	1.64	7.64
528 - 576	86.52	2.02	3.16
592 - 640	84.20	4.41	3.28
656 - 704	88.16	0.88	3.00
720 - 768	86.33	1.90	4.48
784 - 832	85.00	2.22	4.13
848 - 896	84.94	1.82	3.94
912 - 960	84.96	1.48	3.88
Whole tissue +ATP	47.26	31.24	1.60
Whole tissue -ATP	86.57	1.30	5.67
Control	101.8	0	0

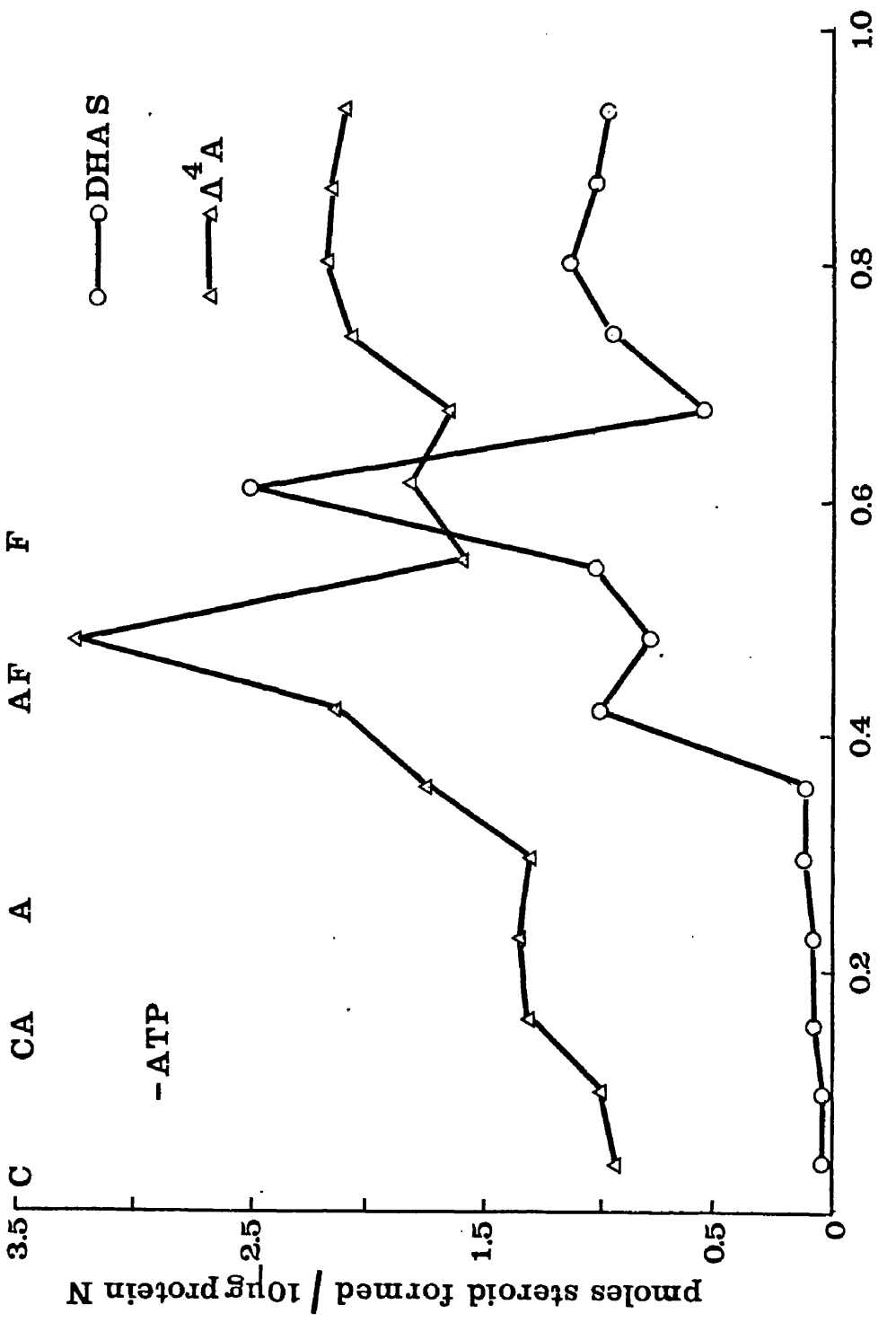


Figure 14. Distribution of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities in the adrenal gland of a previable human foetus (Expt. 9).

DHA sulphokinase activity rose at the adult/foetal zone junction to attain a peak at the edge of the foetal zone, and declined thereafter to levels (0.9 - 2.2%) higher than in the adult zone (0.1 - 0.3%).

Experiment 10. (Table 27; Fig. 15) The conversions of [^{3}H] DHA to its sulphate (maximum 6.6%) were again much lower and to androstenedione higher (maximum 8.7%) than those obtained in the presence of exogenous ATP. DHA sulphokinase activity was low in the adult zone (0.1 - 0.3%) and rose at the adult/foetal zone junction. No peak of this enzyme activity was observed at the edge of the foetal zone. Androstenedione formation attained a marked peak at the adult/foetal zone junction and decreased thereafter. The conversions to this steroid in the foetal zone (1.3 - 1.8%) were greater than at the surface of the gland (0.7 - 0.9%).

The results confirm and extend those obtained in the incubations of whole gland preparations in the absence of ATP, indicating a requirement of ATP for sulphation of DHA by both zones of the foetal adrenal gland and an

Table 27. Metabolism of [7α-^{3}H] DHA by adrenal tissue from a foetus of 23 weeks gestation incubated at tissue:steroid 5,000:1 in the absence of exogenous ATP (Experiment 10)

Distance from gland surface (μm)	Percentage of radioactivity incubated present in isolated steroid		
	DHA	DHAS	Δ^4A
16 - 64	95.59	0.11	0.69
80 - 128	94.57	0.15	0.90
144 - 192	88.86	0.26	2.49
208 - 256	78.40	0.27	8.72
272 - 320	76.70	0.28	8.05
336 - 384	87.65	1.78	1.72
400 - 448	87.12	3.53	1.02
464 - 512	87.50	3.00	1.06
528 - 576	84.42	3.17	1.24
592 - 640	84.75	4.66	1.33
656 - 704	84.23	4.90	1.45
720 - 768	84.08	5.05	1.87
784 - 832	86.30	5.32	1.44
848 - 896	80.58	6.59	1.37
912 - 960	85.85	4.09	1.28
976 -1,024	85.02	4.86	1.29
Whole tissue	88.82	4.73	3.39
Control	95.70	0	0

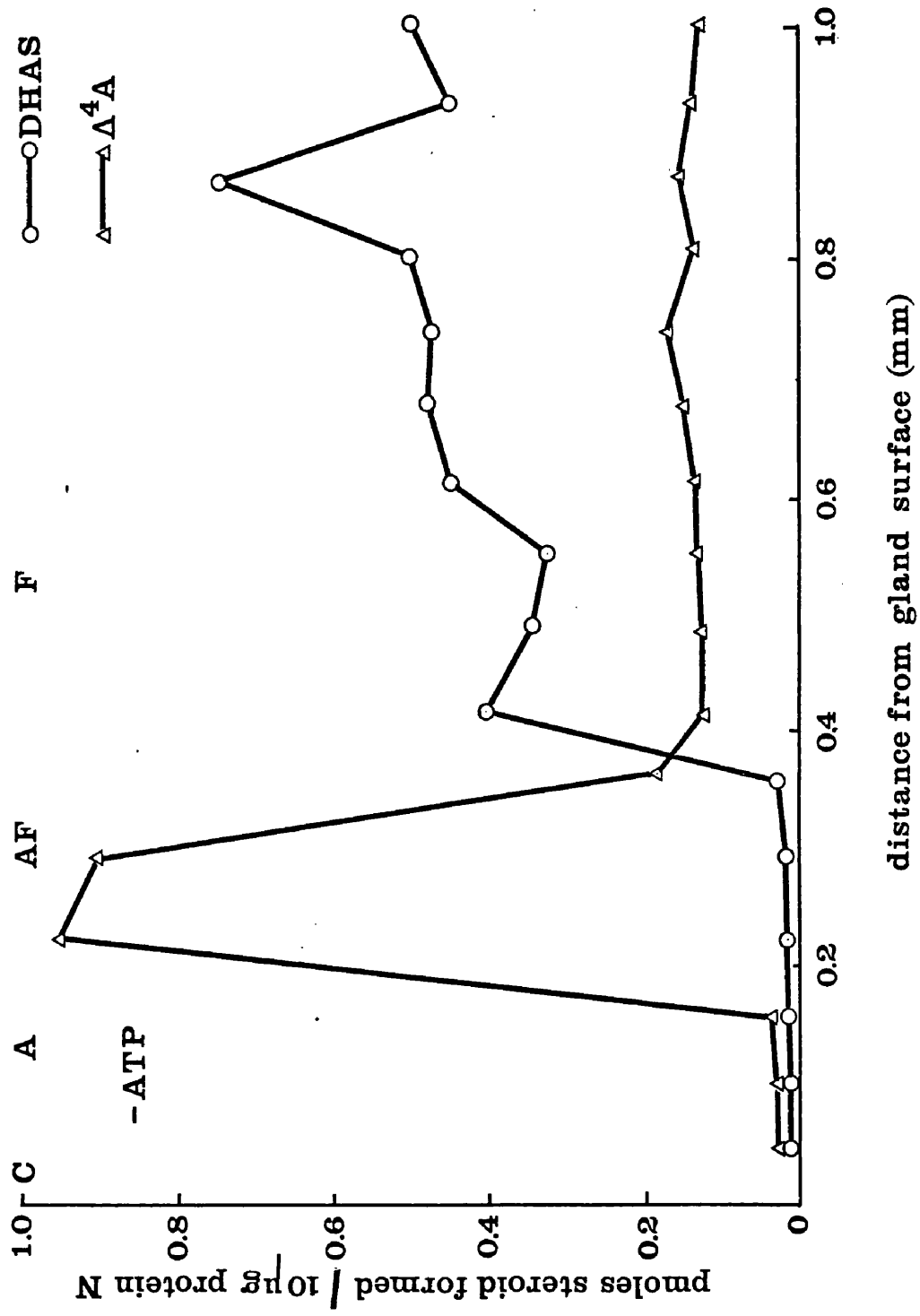


Figure 15. Distribution of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities in the adrenal gland of a previable human foetus (Expt. 10).

increase in androstenedione formation in the absence of high DHA sulphokinase activity. Evidence was obtained for the existence near the adult/foetal zone junction of cells which display enhanced DHA sulphokinase or 3β-hydroxysteroid dehydrogenase-isomerase activities compared with cells from other parts of the glands.

[^{3}H] DHA metabolism in the presence and absence of exogenous ATP by the tissue sections and the whole gland preparations was consistent with what might have been expected on the basis of the relative amounts of adult and foetal zone cells present.

Metabolism of [^{3}H] DHA by adrenal tissue preparations from a newborn female hydrocephalic infant.

The adrenal gland of the newborn female hydrocephalic infant used in experiment 11 (Plate V) had the histological appearance of the gland of a normal human newborn infant, consisting mainly of foetal zone, surrounded by a narrow adult zone.

Adult zone tissue metabolized more [^{3}H] DHA than that from the foetal zone (Table 28). As was found in

Plate V. Adrenal gland of a newborn hydrocephalic infant.

H. & E. (X 150).

Table 28. Metabolism of [7α-^{3}H] DHA by adrenal tissue preparations from a newborn hydrocephalic infant (Experiment 11)

Tissue	Percentage of radioactivity incubated present in isolated steroid			
	DHA	DHAS	Δ^4A	11βΔ^4A
Adult zone	49.02	4.56	32.82	8.23
Foetal zone	60.65	20.90	3.37	2.28
Chopped gland (10,000:1)	4.37	58.24	22.34	1.53
Chopped gland (100,000:1)	10.32	64.14	11.58	0.22
Control	90.77	0	2.87	1.97

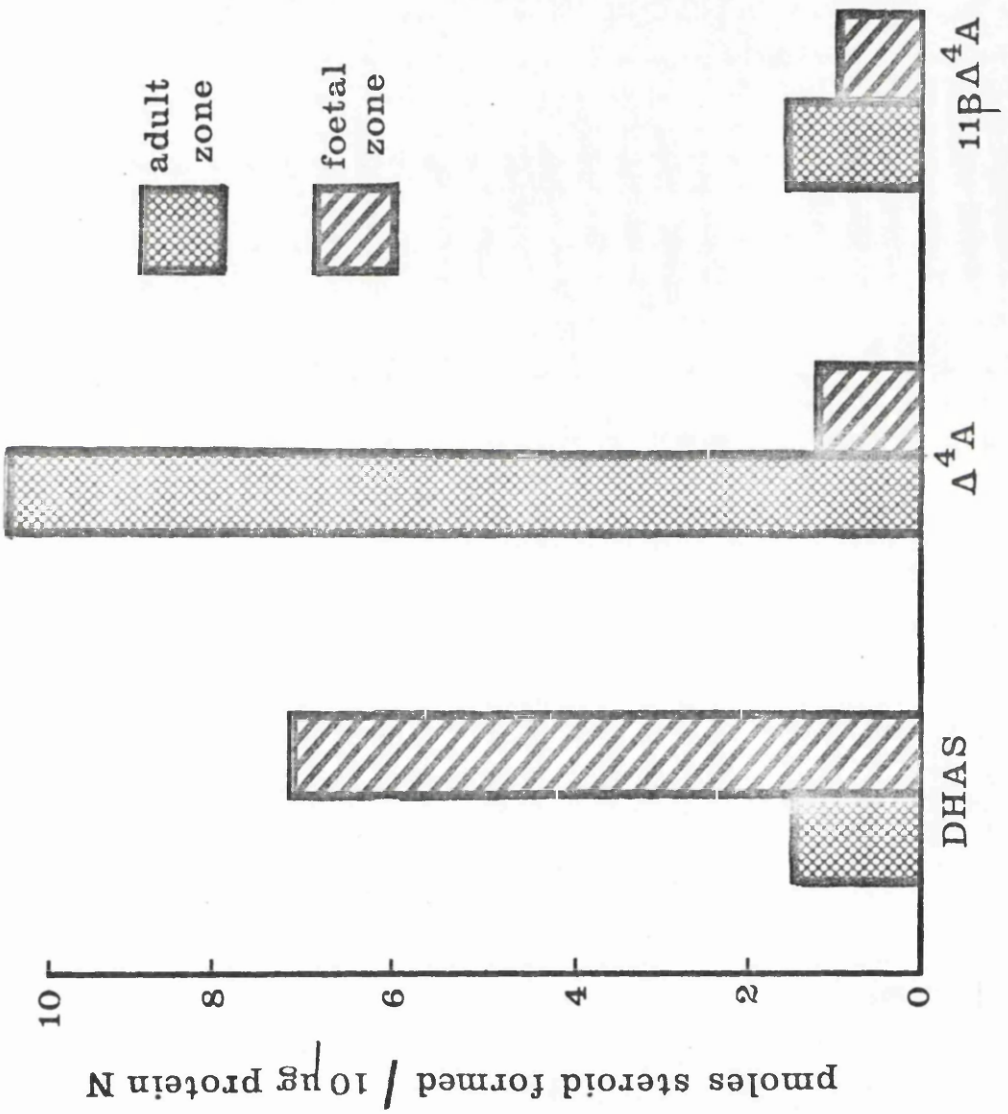


Figure 16. DHA metabolism by histologically-defined adrenal tissue from a newborn female hydrocephalic infant (Expt. 11).

experiment 5, using tissue from the adrenal gland of a previable human foetus, the foetal zone converted approximately five times more [^{3}H] DHA to its sulphate than did the adult zone (Fig. 16). The conversions to DHA sulphate in the two zones (4.6 & 20.9%) were similar to those observed in the midterm foetus (7.2 & 27.5%). The formation of androstenedione, however, was higher than that observed at midterm and was approximately nine times greater in the adult (32.8%) than in the foetal zone (3.4%). Conversions of [^{3}H] DHA to 11β-hydroxyandrostenedione were also greater in the adult (8.2%) than in the foetal zone (2.3%). Formation from [^{3}H] DHA of androstenedione (2.87%) and 11β-hydroxyandrostenedione (1.97%) was, however, obtained in the boiled tissue control incubation. The identification of 11β-hydroxyandrostenedione in the incubation with foetal zone tissue is, therefore, only tentative.

The chopped tissue preparation incubated at tissue: steroid 100,000:1 converted more [^{3}H] DHA to DHA sulphate and less to androstenedione than that incubated at 10,000:1, confirming the results obtained with tissue from previable foetuses. Conversions to 11β-hydroxyandrostenedione by

the chopped tissue were less than that obtained in the control incubation. The formations of DHA sulphate at both tissue:steroid ratios (58 & 64%) were much greater than in the adult (4.6%) and foetal zone (21%).

Metabolism of [^{3}H] DHA by histologically-defined tissue from the adrenal gland of a newborn female anencephalic infant of 42 weeks gestation.

The adrenal gland of the newborn post-mature anencephalic infant used in experiment 12 (Plate VI) consisted almost entirely of adult zone with some medullary and residual foetal zone tissue. The adult zone had a much more differentiated histological appearance than usually observed in anencephaly (c.f. Plate II), consisting of three distinct zones which will be referred to as the outer dark, clear and inner dark zones. Tissue sections were obtained from the three adult zones and from the junction between the inner dark and foetal zones.

a. In the presence of exogenous ATP (Table 29; Fig. 17)

Conversions of [^{3}H] DHA to its sulphate increased with increased depth in the gland. Sections from the surface of the

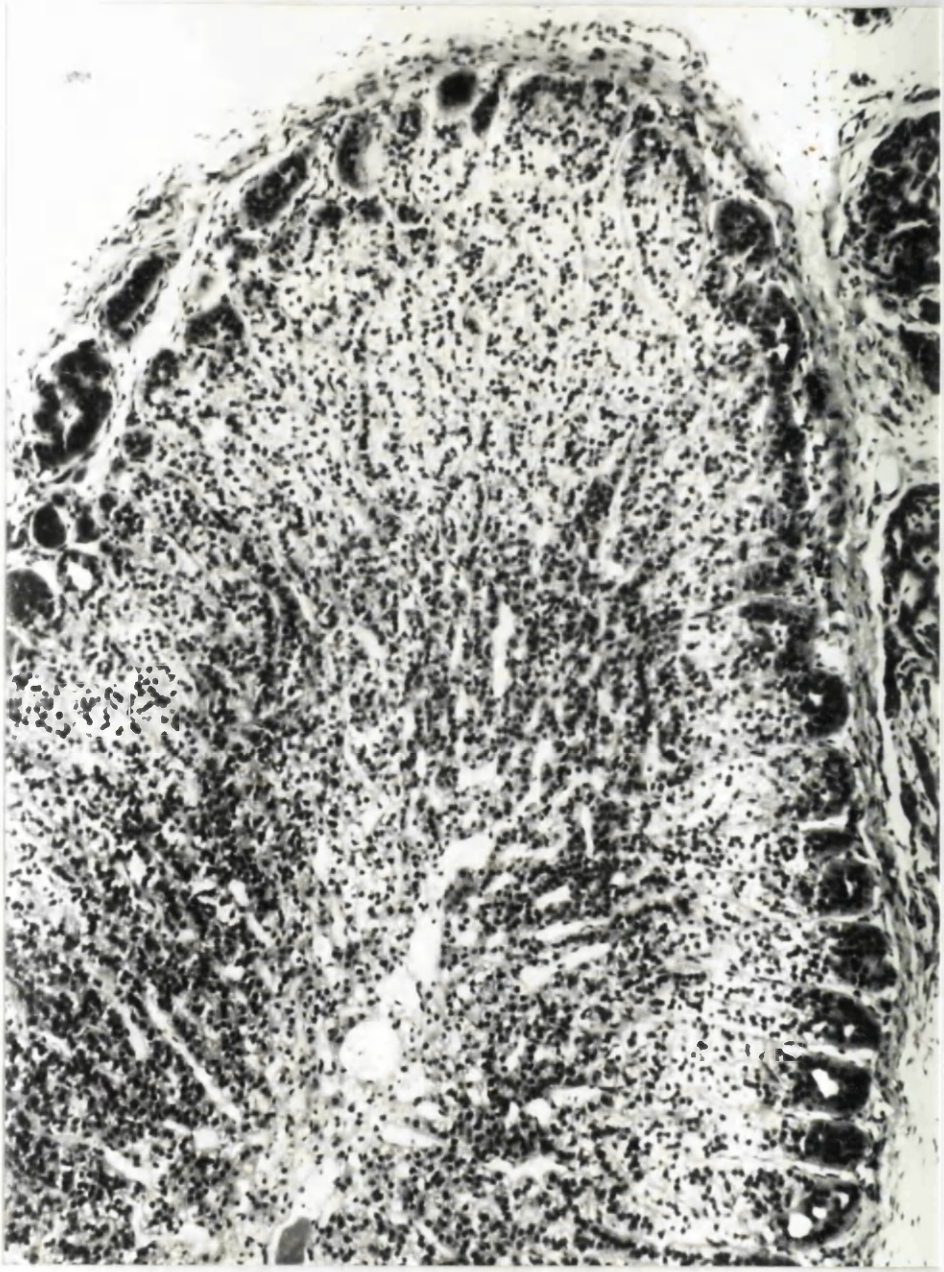


Plate VI. Adrenal gland of a newborn post-mature anencephalic infant.
H. & E. (X 140).

Table 29. Metabolism of [7α-^{3}H] DHA by adrenal tissue from a newborn anencephalic infant of 42 weeks gestation incubated at tissue: steroid 10,000:1 in the presence and absence of exogenous ATP (Experiment 12)

Distance from gland surface (μm)	Histology +	Percentage of radioactivity incubated present in isolated steroid			
		DHA	DHAS	Δ^4A	11βΔ^4A
16 - 64	Cap./O.D.	55.19	0.61	48.52	0.82
80 - 128 *	O.D.	36.74	0.58	69.35	0.93
144 - 192	O.D.	16.38	23.63	50.77	1.03
400 - 448 *	Clear	60.33	1.88	44.88	1.60
528 - 576	Clear	13.00	23.41	59.32	2.71
656 - 704 *	Clear/I.D.	63.57	1.30	32.13	1.26
784 - 832	I.D.	14.59	32.01	36.81	0.51
912 - 960 *	I.D.	57.40	1.48	37.88	0.73
1040-1088	I.D./Foetal	17.72	40.19	27.79	0.86
1168-1216 *	I.D./Foetal	59.44	1.88	26.64	1.42
Whole tissue		42.15	12.51	50.13	1.49
Whole tissue *		38.79	1.00	64.15	3.31
Blank		102.9	0	0	0

* incubated in the absence of exogenous ATP

\+ Cap., capsule; O.D., outer dark; I.D., inner dark.

Figure 17. DHA metabolism by histologically-defined adrenal tissue from a newborn anencephalic infant incubated in the presence of exogenous ATP (Expt.12).

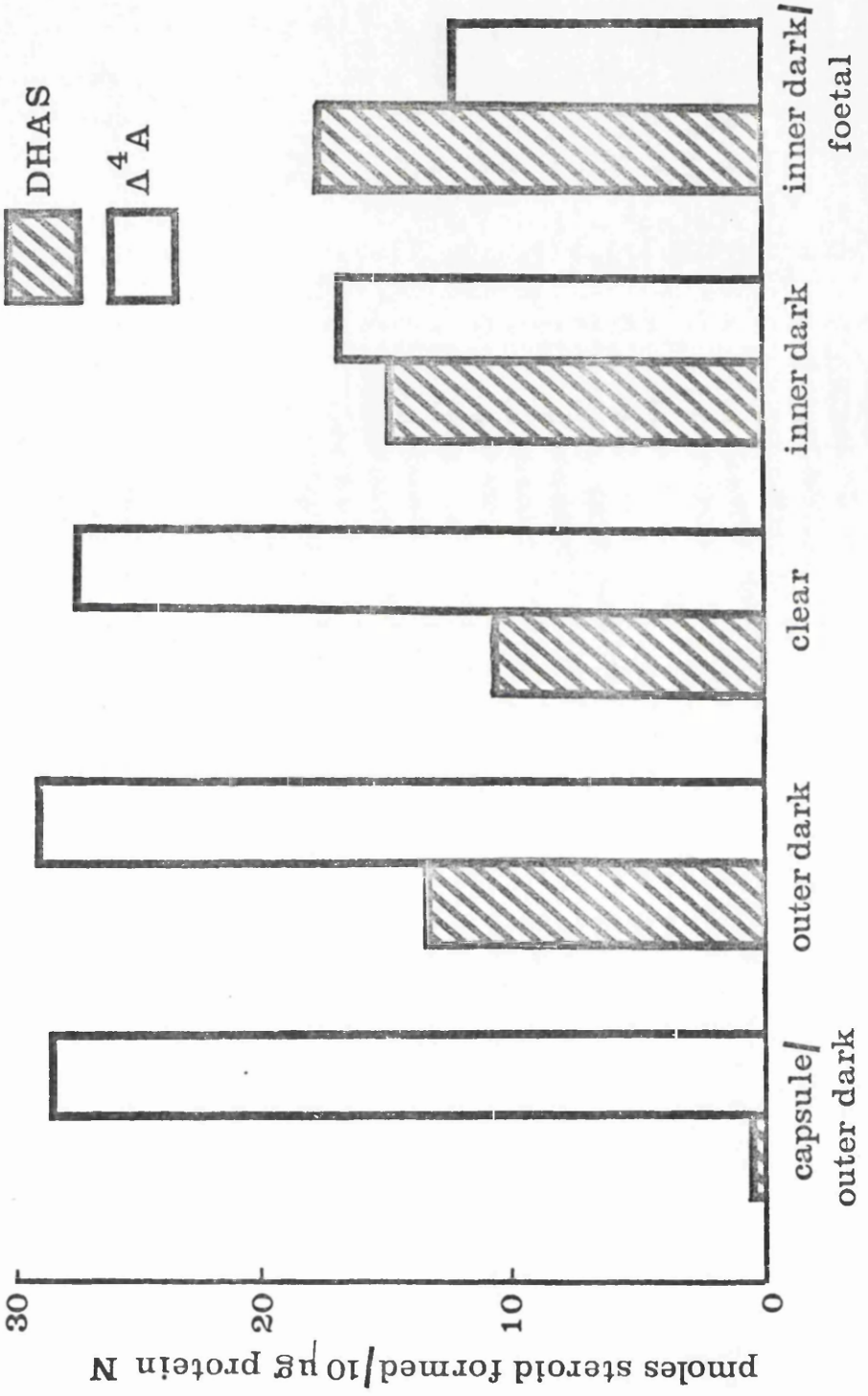
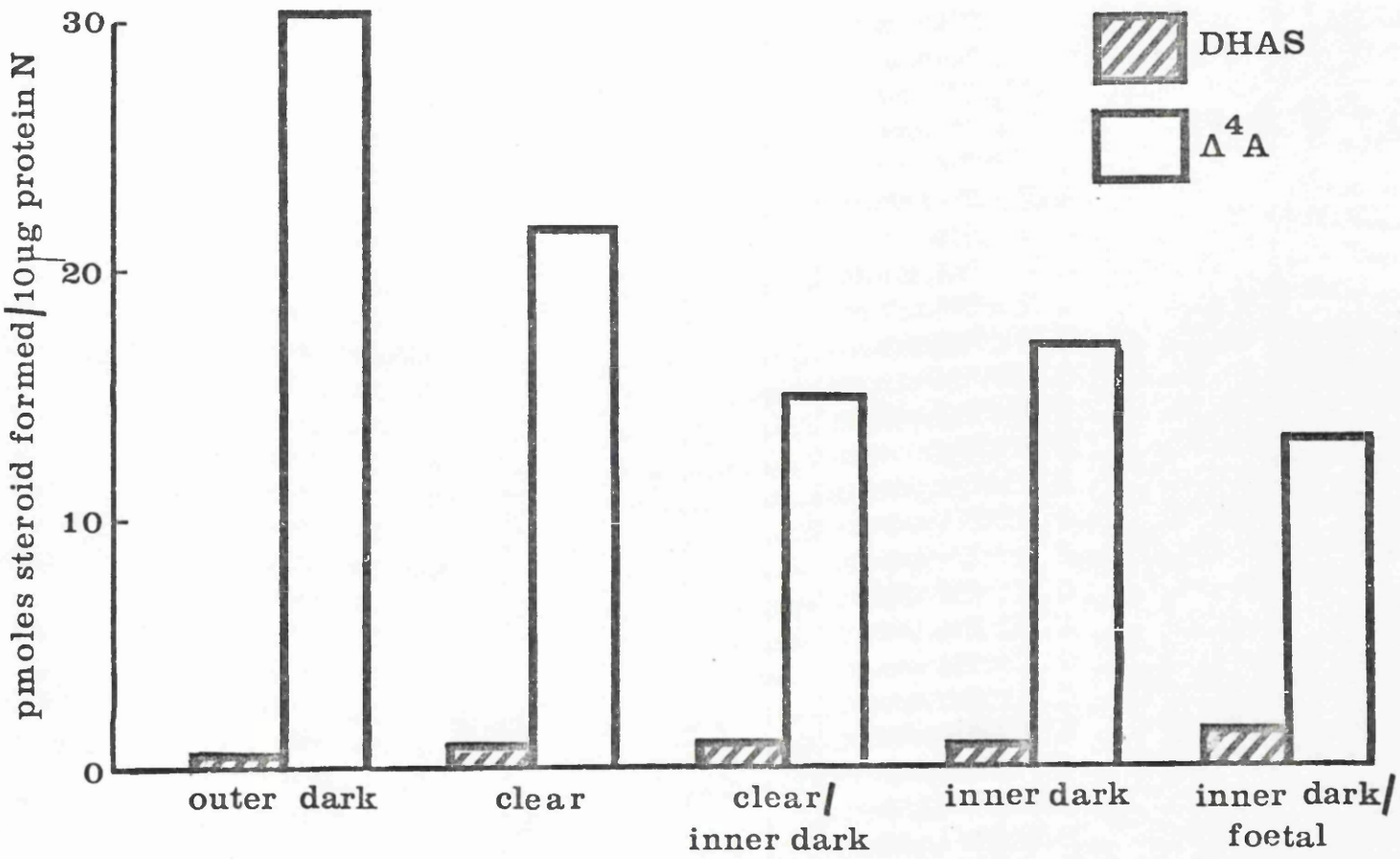


Figure 18. DHA metabolism by histologically-defined adrenal tissue from a newborn anencephalic infant incubated in the absence of exogenous ATP (Expt.12).



gland, which were composed almost entirely of capsular tissue, formed very little DHA sulphate (0.6%), but converted 49% of the [^{3}H] DHA to androstenedione. Conversions to DHA sulphate were 23 - 32% in the adult zones and 40% in the mixed inner dark and foetal zone sections. Formation of androstenedione in the adult zones (37 - 59%) was higher than observed in the adult zone of the newborn hydrocephalic infant (33%). The lowest conversion to androstenedione occurred in the sections containing foetal zone cells. Conversions to 11β-hydroxyandrostenedione (0.5 - 2.7%) were much lower than to androstenedione.

b. In the absence of exogenous ATP (Table 29; Fig.18).

Omission of ATP from the incubation medium, as was observed with adrenal tissue from previable foetuses, resulted in markedly lower conversion of [^{3}H] DHA to its sulphate (0.6 - 1.9%). Androstenedione formation was increased in the outer dark zone, reduced in the clear zone and unchanged in the inner dark and mixed inner dark and foetal zone sections. Conversions to 11β-hydroxy-

androstenedione were similar to those obtained in the presence of exogenous ATP.

The whole gland homogenate, as in the experiments with adrenal tissue from previable foetuses, metabolized less [^{3}H] DHA to its sulphate and more to androstenedione than in the presence of exogenous ATP. The patterns of DHA metabolism by the homogenates were consistent with those in the corresponding incubations with tissue sections.

The adult zones of the adrenal gland of the newborn post-mature anencephalic infant possessed, therefore, greater DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities than the corresponding zone in the glands of the newborn hydrocephalic infant or the previable foetuses.

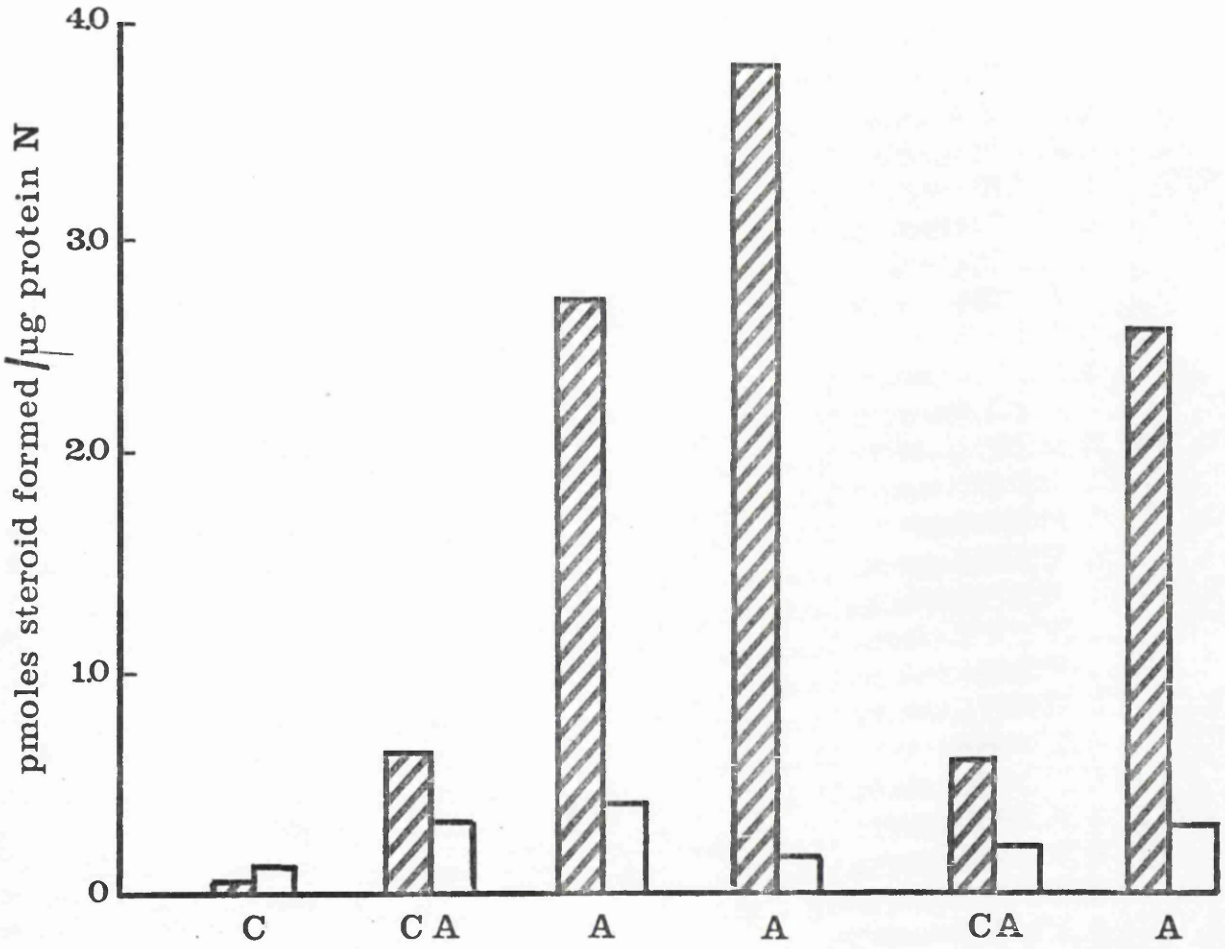
Metabolism of [^{3}H] DHA by adult zone tissue from the adrenal gland of a newborn female anencephalic infant of 38 weeks gestation (Table 30; Fig. 19).

The adrenal tissue from the newborn anencephalic infant delivered by Caesarean section used in experiment 13 had the histological appearance of adrenal glands

Table 30. Metabolism of [7α-^{3}H] DHA by adult zone tissue from the adrenal gland of a newborn anencephalic infant of 38 weeks gestation incubated at tissue:steroid 10,000:1

Distance from gland surface (μm)	Histology	Percentage of radioactivity incubated present in isolated steroid			
		DHA	DHAS	Δ^4A	11βΔ^4A
16 - 64	Almost entirely capsule	96.03	0.70	2.00	0.11
144 - 192	Mainly capsule	74.18	12.66	6.20	0.32
272 - 320	Mainly adult zone	37.04	54.32	7.70	0.24
400 - 448	Adult zone	10.05	82.65	2.98	0.22
16 - 64	Mainly adult zone	80.32	11.98	4.16	0.15
144 - 192	Adult zone	27.84	53.56	6.53	0.15
Whole tissue +ATP		20.41	64.29	5.74	0.22
Whole tissue -ATP		69.65	8.12	11.12	0.36
Blank		99.03	0	0	0

Figure 19. DHA metabolism by histologically-defined adrenal tissue from a newborn anencephalic infant (Expt. 13).



typically found in anencephaly, consisting mainly of adult zone tissue with some residual foetal zone tissue. In contrast to the gland used in experiment 12, little differentiation of the adult zone was evident. Tissue sections containing capsule and/or adult zone cells were prepared from two bores of the gland.

Capsular tissue containing a few cells from the adult zone converted only 0.7% of the [^{3}H]DHA to DHA sulphate. Sulphation increased with increased depth in the gland and the maximum conversion to DHA sulphate in adult zone tissue free from cells of the capsule was 83%. Conversions to androstenedione (2.0 - 7.7%) also increased with increased depth in the gland. Little [^{3}H] DHA was converted to 11β-hydroxyandrostenedione (0.1 - 0.4%).

Omission of ATP from the medium in incubations with the tissue homogenates markedly decreased formation of DHA sulphate and increased conversion to androstenedione. The metabolism of [^{3}H] DHA in the presence of ATP by the homogenate was consistent with that observed in the incubations with tissue sections and resembled more that obtained with the previable foetuses than with the newborn hydrocephalic and post-mature anencephalic infants.

The DHA sulphokinase activity in the adult zone was, however, much greater than in the corresponding zone in the younger foetuses.

DISCUSSION

The identification of isotopically-labelled steroids.

The availability of isotopically-labelled steroids of high specific radioactivity, such as the [^{3}H] DHA used in the present study (500 mCi/mmole and 12.9 Ci/mmole), permits their incubation with tissue at concentrations approximating to those pertaining *in vivo*. It is rarely possible, however, in such experiments, to isolate sufficient quantities of isotopically-labelled metabolites for their detection by conventional analytical procedures (see review by Brooks *et al*, 1970). The techniques of Berliner and Salhanick (1956) provide a convenient method for the identification and characterization of microquantities of isotopically-labelled steroids. These authors considered the possibility that a steroid and its derivatives might be isopolar with radioactive contaminant and its corresponding derivatives to be remote. Berliner and Salhanick recommended the formation of at least two derivatives of each steroid to eliminate the possibility of small amounts of extraneous radioactivity being carried with the authentic substances during chromatography. In experiments 5 and 11 of the present study, the specific

activities of each isolated steroid, after initial fractionation and purification procedures, and two of its derivatives were in agreement ± 5% of the mean values. The constancy of these sequentially determined specific activities indicated an absence of radioactive contaminants in the extracts and probably reflects the purification of the [^{3}H] DHA immediately prior to incubation and the limited number of biochemical transformations which DHA underwent in the adrenal tissue of the human foetus and newborn infant. In the other experiments of the present study, therefore, the constancy of the specific activities of the isolated steroids and one derivative was considered adequate proof of radiochemical purity.

Isotopically-labelled steroids may also be identified by recrystallization to constant specific activity with the pure unlabelled steroids (Axelrod, **Matthijssen**, Goldzieher and Pulliam, 1965). It is considered unlikely that a radioactive contaminant will be both isopolar and isomorphous with the authentic steroid. In experiments 6 and 12 of the present study, steroids, purified by chromatography and derivative formation, were diluted with further carrier

steroid and subjected to recrystallization procedures. The constancy of the specific activities of the crystals with those of the mother liquors in every case was considered additional evidence that the techniques of chromatography and formation of one derivative were sufficiently rigorous to establish radiochemical purity of the isotopically-labelled steroids isolated following incubation of $[^{3}H]$ DHA with adrenal tissue in the present experiments.

The metabolism of [^{3}H] DHA by whole adrenal gland preparations from previable human foetuses.

In agreement with the results of other workers, the whole adrenal gland preparations from previable foetuses used in the present study converted [^{3}H] DHA to DHA sulphate (Villee and Loring, 1968) and androstenedione (Bloch, Tissenbaum, Rubin and Deane, 1962; Sulcova, Capcova, Jirášek and Starka, 1969; Villee and Loring, 1969). In every case DHA sulphate was the principal metabolite of [^{3}H] DHA at tissue:steroid 10,000:1 in the presence of exogenous ATP and was formed in much larger quantities than androstenedione. The results of the time-course experiments indicate that sulphoconjugation of [^{3}H] DHA was much more rapid than its conversion to androstenedione.

Not all of the incubated radioactivity was accounted for as DHA sulphate, androstenedione or unmetabolized DHA in some of the present experiments, suggesting the conversion of [^{3}H] DHA to other metabolites. 7-hydroxy-DHA (Jirásek, Sulcova, Capcova, Röhling and Starka, 1969) and testosterone (Jirásek et al., 1969; Villee and Loring, 1969) have been reported as metabolites of DHA by foetal adrenal glands in vitro. Benagiano, Mancuso, Mancuso, Wiqvist, and Diczfalusy (1969) reported the isolation of testosterone and testosterone

sulphate, and Mancuso, Benagiano, Dell'Acqua, Shapiro and Diczfalusy (1968) the isolation of 11β-hydroxyandrostenedione and 11β-hydroxytestosterone from adrenal glands of previable foetuses perfused with androstenedione. No radioactivity was detected by radiochromatogram scanning of the solvolysed extracts in the present experiments other than in association with solvolysed DHA sulphate. Evidence was obtained, however, by radiochromatogram scanning for the presence in the free steroid extracts of radioactivity in association with compounds more polar than DHA. It is possible, therefore, that small amounts of the above mentioned and perhaps other metabolites of DHA were formed in the present experiments. Villee & Loring (1969) reported the presence of a low, but detectable, steroid sulphatase activity with DHA sulphate as substrate in incubations of foetal adrenal glands. This was not substantiated in the present experiments since no decrease in conversions to DHA sulphate occurred during incubation periods of up to 2 h duration. Cooke, Cowan, and Taylor (1970) incubated a mixture of [^{14}C] pregnenolone and [^{3}H] pregnenolone sulphate with homogenates of foetal adrenal glands. No tritium could be detected in unconjugated pregnenolone, 17α-hydroxypregnenolone or DHA, indicating the absence of any significant steroid

sulphatase activity. Neither do the results of the present experiments support the suggestion of Villee and Villee (1964) that short incubation periods with homogenized foetal adrenal glands favour the accumulation of sulphated and other polar steroids.

The failure to detect conversion of [^{3}H] DHA to 11β-hydroxyandrostenedione in experiment 5 is in agreement with the report of Bloch et al, (1962) but at variance with the results of Jirásek et al, (1969). This steroid has been identified in extracts of foetal adrenal glands by Bloch and Benirschke (1956) and was isolated in considerable quantities from the adrenal glands of previable foetuses perfused with androstenedione (Mancuso et al, 1968). Milner and Mills (1970) reported that the amounts of 11β-hydroxyandrostenedione formed from [^{3}H] pregnenolone by slices of foetal adrenal glands increased between 2 and 5 h and 24 h of incubation. The failure to detect this metabolite in the present study may, therefore, result from the low 3β-hydroxysteroid dehydrogenase-isomerase activity and/or the shortness of the incubation period used (30 min).

The low in vitro activity of 3β-hydroxysteroid dehydrogenase-isomerase observed in the adrenal gland of the previable

human foetus is in agreement with the reports of Bloch and Benirschke (1962), Sulcova et al., (1968) and Villee and Loring (1969). This low activity could result from a deficiency in the amount of enzyme present, inhibition of the enzymic activity or competition for available substrate by other enzyme systems.

The results of the incubations with [^{3}H] DHA at different tissue:steroid ratios indicate that, while DHA sulphate was the principal metabolite formed in every case, the ratio of androstenedione to DHA sulphate formed decreased when the amount of tissue, and consequently the amount of enzymes, incubated was increased. Conversion of DHA to androstenedione would appear, therefore, to increase once the DHA sulphokinase system is saturated with substrate, suggesting a deficiency in the amount of 3β-hydroxysteroid dehydrogenase-isomerase relative to the amount of DHA sulphokinase enzyme present.

Placental progesterone and oestrogens have been suggested as possible inhibitors of foetal adrenal 3β-hydroxysteroid dehydrogenase-isomerase activity (Bloch, 1968). Villee (1966) observed a decrease in this enzyme activity using pregnenolone as substrate in foetal adrenal

glands in organ culture if progesterone was introduced into the culture medium. Inhibition of adrenal 3β-hydroxysteroid dehydrogenase-isomerase activity in the adult rat by oestrogens has been reported by Goldman (1968). The foetal adrenal gland is capable of extensive hydroxylation of progesterone and of hydroxylation and sulphoconjugation of oestrogens (see Tables 1 & 2). The preincubation of foetal adrenal tissue prior to the addition of [^{3}H] DHA might, therefore, be expected to have effected metabolism and, perhaps, inactivation of these steroids. Preincubation of adrenal tissue for 30 minutes had little effect, however, on the formation of androstenedione from [^{3}H] DHA in the present study. The low activity of 3β-hydroxysteroid dehydrogenase-isomerase would not appear, therefore, to be due to inhibition by steroids of extra-adrenal origin.

ATP is a precursor of 3'-phosphoadenosine 5'-phosphosulphate ("active sulphate") which is a reactant in the sulphoconjugation of alcoholic and phenolic compounds in mammals (Robbins and Lipmann, 1956). The steroid sulphokinases involved in the production of C-21 steroid sulphates by slices of adrenal glands of newborn infants are highly dependent on

the availability of this substance (Klein and Giroud, 1965). Villee and Loring (1969) have reported a requirement of ATP for the sulphoconjugation of DHA and pregnenolone by homogenized human foetal adrenal glands. The omission of ATP as an added co-factor in the present experiments resulted in markedly lowered DHA sulphate formation. The lower levels of sulphoconjugation were accompanied by increased conversions of [^{3}H] DHA to androstenedione, suggesting competition for substrate between the 3β-hydroxysteroid dehydrogenase-isomerase and DHA sulphokinase systems in the presence of exogenous ATP. The increases in androstenedione formation were, however, much smaller than the decreases in conversions to DHA sulphate. The greatly reduced metabolism of [^{3}H] DHA in the absence of extensive sulphoconjugation is consistent with a deficiency of 3β-hydroxysteroid dehydrogenase-isomerase in the adrenal gland of the previable human foetus.

Koritz (1964) reported that 3β-hydroxysteroid dehydrogenase activity is inhibited by NADH and NADPH. Enhanced activities of this enzyme system were obtained in mouse testis homogenates incubated in a nitrogen atmosphere with only a NAD regenerating system present compared with incubation in the presence of

both NAD and NADPH regenerating systems (Samuels, Matsumoto, Aoshima and Bedrak, 1969). The incubation of foetal adrenal tissue under nitrogen in medium containing NAD as the only added cofactor in the present study at the three tissue:steroid ratios investigated did not reveal any increases of androstenedione formation greater than were observed when ATP alone was omitted from the incubation medium. The absence of any significant rise in 3β-hydroxysteroid dehydrogenase-isomerase activity under favourable *in vitro* conditions is interpreted as further evidence of a deficiency of this enzyme system in the adrenal gland of previable human foetuses.

The detection of low 3β-hydroxysteroid dehydrogenase-isomerase activity in the present study is at variance with the results of Bloch *et al.*,(1962) who obtained comparatively high conversions of DHA to androstenedione with adrenal glands of foetuses of 9 to 19 weeks gestation. Their results, however, might be explained by the low tissue: steroid ratios employed (250 - 1,500:1) and the absence of ATP from their incubation medium. The detection of activity of this enzyme system is also in disagreement with the result of perfusion studies (Bolté *et al.*, 1966) in which no

conversion of DHA and pregnenolone to Δ^4-3-oxosteroids was detected in the adrenal gland of human foetuses at midgestation. Diczfalusy (1969) has suggested that loss of substrate specificity of enzyme systems may occur during preparation of tissue for *in vitro* studies and in support of this theory has cited the conversions of perfused 17α-hydroxypregnenolone (Jackanicz *et al.*, 1969), 17α, 21-dihydroxypregnenolone (Pasqualini *et al*, 1968), and 21-hydroxypregnenolone (Pasqualini *et al*, 1970) to Δ^4-3-oxosteroids by previable human foetuses. The recent perfusion studies of Telegdy *et al*.,(1970) have, however, demonstrated that the results of perfusion studies, as of *in vitro* studies, must not too readily be extrapolated to the *in vivo* situation. These authors perfused a mixture of [^{3}H]cholesterol and [^{14}C]acetate into previable foetuses and the foeto-placental unit at midgestation and isolated pregnenolone, DHA, progesterone and androstenedione from the foetal adrenal glands. The last-named steroid, was however, formed from acetate but not cholesterol, suggesting that perfused steroids may not be metabolized similarly to steroids synthesized endogenously.

The isolation of progesterone and androstenedione in these experiments is the first report of their formation from perfused precursors other than Δ^4-3-oxosteroids. Previous perfusion studies were, however, conducted at lower temperature ($20^{\circ}C$) and lower flow rate than used by Telegdy *et al.*,(1970). Solomon *et al.*,(1967) and Coutts and MacNaughton (1969) concluded that neither perfused acetate nor cholesterol were efficient steroid precursors in previable human foetuses, whereas Telegdy *et al.*, (1970) proposed that the foetal adrenal gland represents an active site of cholesterol metabolism and, perhaps, its biosynthesis within the foeto-placental unit at midgestation. The repetition of other perfusion studies under more physiological conditions may also yield different results. The levels of DHA and its sulphate in the adrenal glands of newborn human infants are 5.8 and 1.30 ug/10g tissue (Matsumoto *et al.*, 1968) which represent tissue:steroid ratios of approximately 1,700,000:1 and 800,000:1 respectively. The results of the present study indicate an inverse relationship between tissue:steroid ratio and androstenedione formation. The 3β-hydroxysteroid dehydrogenase-isomerase activity obtained at tissue:steroid 10,000:1 in the present study may be greater, therefore, than that

occurring *in vivo*.

Milner and Mills (1970) reported that the formation from pregnenolone of DHA sulphate relative to DHA and of cortisol and 11β-hydroxyandrostenedione from progesterone increased between 12 and 27 weeks gestation in slices of foetal adrenal glands. No correlation of DHA metabolism with gestational age was obtained in the present study. Villee and Villee (1964) have, however, reported different patterns of pregnenolone metabolism by adrenal tissue from twin foetuses incubated under identical conditions. It appears, therefore, that wide variations in steroid metabolism may occur in adrenal tissue of the same maturity.

It may be concluded from the present study that the adrenal gland of the human foetus during the second trimester of pregnancy possesses a high activity of DHA sulphokinase and a deficiency of 3β-hydroxysteroid dehydrogenase-isomerase.

The effect of freezing of tissue on [^{3}H] DHA metabolism.

The microtechnique of Grunbaum *et al.*,(1956), which was used in the present study to obtain histologically-defined foetal adrenal tissue, involves freezing of the adrenal glands to -15°C. It was necessary, therefore, to determine

if this procedure had any affect on the metabolism of [^{3}H] DHA. No alteration in the formation of androstenedione and DHA sulphate was observed using foetal adrenal tissue which had been frozen in solid CO_2 for 2 h prior to homogenization compared with tissue which had been homogenized after storage in ice at 0 - 4°C for 30 min. Bloch et al., (1962) observed no loss of 3β-hydroxysteroid dehydrogenase-isomerase activity in foetal adrenal glands stored at -15°C for up to two weeks.

The metabolism of [^{3}H] DHA by adult and foetal zone tissue from the adrenal gland of a previable human foetus.

Both zonal fractions prepared from the adrenal gland of a foetus of 22 weeks gestation converted [^{3}H] DHA to androstenedione and DHA sulphate. Adult zone tissue metabolized less of the incubated [^{3}H] DHA than did the foetal zone. This is consistent with the report of Johannisson (1968) that, whereas the foetal zone showed ultrastructural features indicative of secretory activity during the first trimester of pregnancy, there was little evidence of steroidogenic activity in the adult zone until the last part of the second and the beginning of the third trimester.

Histochemists have presented conflicting reports of the distribution of 3β-hydroxysteroid dehydrogenase activity within the foetal adrenal gland. Goldman et al., (1964) and Cavallero and Magrini (1966) detected this enzyme activity in the adult zone of foetuses obtained after the fourth month of gestation but found that activity was minimal to absent in the foetal zone at all stages of gestation. Jirásek et al.,(1969) observed activity in the **foetal zone** of glands from foetuses of 20 - 50 days and 2 - 3 months gestation but not in the adult zone until the fourth month of gestation. Bloch et al.,(1962) and Niemi and Baillie (1965) reported the presence of activity in both zones of the foetal adrenal gland. 3β-Hydroxysteroid dehydrogenase-isomerase activity has been demonstrated biochemically in both zones of the adrenal gland of the foetus of 22 weeks gestation used in the present study, and has been found to be greater in the adult than in the foetal zone. DHA sulphokinase activity was also observed in both zones of the gland and was greater in the foetal than in the adult zone. The presence in the foetal zone, which occupies the bulk of the foetal adrenal gland, of higher DHA sulphokinase and lower 3β-hydroxysteroid dehydrogenase-

isomerase activities than in the adult zone, is consistent with the presence in the human foetus at midgestation of large amounts of Δ^5-3β-hydroxysteroids and sulphoconjugated steroids (see review by Mitchell, 1967).

Metabolism of [^{3}H] DHA to 11β-hydroxyandrostenedione was not detected in either zone of the foetal adrenal gland studied. Bloch and Benirschke (1962) obtained conversion of acetate to this steroid using whole adrenal gland slices but not with slices containing only foetal zone tissue.

<u>The quantitative histological distribution of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities in the adrenal glands of previable human foetuses</u>.

The distributions of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activities observed in the glands of previable foetuses incubated in the presence of exogenous ATP confirm and extend the results obtained using zonal tissue fractions of the adrenal gland of the foetus of 22 weeks gestation. 3β-Hydroxysteroid dehydrogenases-isomerase activity was slightly greater in the adult than in the foetal zone and DHA sulphokinase activity was restricted to,

in the main, the cells of the latter zone. In the absence of exogenous ATP, markedly reduced conversions of [^{3}H] DHA to its sulphate were obtained. The distribution of DHA sulphokinase within the glands was, however, similar to that observed in the presence of the cofactor. The omission of ATP from the incubation medium altered the distribution of 3β-hydroxysteroid dehydrogenase-isomerase activity. Conversions of [^{3}H] DHA to androstenedione were greater in the foetal than in the adult zones of the glands, suggesting that the levels of 3β-hydroxysteroid dehydrogenase-isomerase in the foetal zone may be higher than in the adult zone. This would be consistent with the more differentiated histological appearance of the foetal zone during the first half of pregnancy (Johannisson, 1968). The results of the present experiments suggest that the lower activity of 3β-hydroxysteroid dehydrogenase-isomerase observed in the foetal zone compared with the adult zone of the adrenal glands of previable foetuses incubated in the presence of exogenous ATP reflects substrate competition with the active DHA sulphokinase system present in the former zone. The distribution of 3β-hydroxysteroid dehydrogenase-isomerase activity obtained in the absence of added ATP probably represents more accurately the amounts of this

enzyme system present, whereas the results obtained in the presence of exogenous ATP may reflect more closely the distribution of this enzyme activity in the foetal adrenal gland *in vivo*.

The peaks of androstenedione formation obtained at the adult/foetal zone junction in the absence of added ATP, suggest that certain cells in this region of the adrenal gland of the previable human foetus possess higher levels of the 3β-hydroxysteroid dehydrogenase-isomerase system than are present in other parts of the gland. Niemi and Baillie (1965) observed diformazan deposition in the inner half of the adult zone and in the outermost foetal zone in a histochemical study of 3β-hydroxysteroid dehydrogenase-isomerase activity in the adrenal glands of foetuses obtained during the second trimester of pregnancy.

Distinct peaks of DHA sulphokinase activity were obtained near the adult/foetal zone junction of the adrenal glands of three of the five previable human foetuses used in the present study. Cooke and Taylor (1970) observed considerable sulphokinase activity at the adult/foetal zone junction of adrenal glands of previable foetuses incubated with [^{3}H] pregnenolone and concluded that this region of the glands,

in addition to the foetal zone may be an important site of DHA sulphate biosynthesis from pregnenolone.

The peaks of androstenedione and DHA sulphate formation obtained in experiment 9 (Fig. 14) were not coincident. It is also of interest that the "trough" between the peaks of DHA sulphate formation in experiment 8 (Fig. 13) was the site of a slight peak of androstenedione formation. These observations are consistent with some degree of substrate competition between the 3β-hydroxysteroid dehydrogenase-isomerase and DHA sulphokinase enzyme systems.

Johannisson (1968) reported that the cells of the adult zone merge with those of the foetal zone in an "indistinct transitional zone". The cells in this part of the adrenal glands, during the first trimester, contained greater amounts of smooth and rough-surfaced endoplasmic reticulum, a more developed Golgi complex and more mitochondria than the cells of the adult zone, but were less well differentiated than the cells of the foetal zone. In the first weeks of the second trimester, "dark cells" appeared in the "transitional zone". These cells exhibited a great variation in their electron density and in the

appearance of their smooth endoplasmic reticulum. The variations in electron density were due to increased amounts of smooth endoplasmic reticulum and/or the density of the cytoplasmic matrix. The absence of these "dark cells" from adrenal glands obtained prior to the tenth week of pregnancy and their location between the adult and foetal zones suggests that their presence is not artefactual. Johannisson found that the "dark cells" were visible under the light microscope and had a high affinity for toluidine blue. The failure to detect such cells in the sections obtained in experiments 7 - 10 in the present study which were stained with toluidine blue may be due to the use of frozen sections and/or the thickness of the sections prepared (16 μm; approximately twice the thickness of normal histological sections). It is not, therefore, possible to relate the increased enzyme activities observed near the adult/foetal zone junction of the gland used in the present study to the ultrastructural variations noted in the cells of the "transitional zone" of Johannisson. Griffiths and Glick (1966) have reported a peak of 11β-hydroxylase activity at the fasciculata/reticularis junction of the adrenal cortex of adult rats

treated with ACTH. Grant (1968) has suggested that this may be a result of a slowing of the blood flow through the glands by the longitudinal smooth muscle bundles which might permit greater stimulation by ACTH of the cells at the interface between the fascicular and reticular zones. In the absence of adequate information concerning the nature of the trophic stimulus to the human foetal adrenal gland (see Introduction), no parallel can be drawn, however, with the peaks of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activity observed near the adult/foetal zone junction of the adrenal glands of previable human foetuses observed in the present study.

The metabolism of [^{3}H] DHA by adrenal tissue from a newborn hydrocephalic infant.

The histological appearance of the adrenal gland of the newborn hydrocephalic infant used in experiment 11 was similar to that of a normal newborn infant, suggesting an absence of damage to the foetal hypothalamo-pituitary axis (Benirschke, 1956). The steroidogenic function of this gland was probably, therefore, similar to that of a normal newborn infant.

The adult zone of the adrenal gland of the newborn hydrocephalic infant metabolized much more of the incubated [^{3}H] DHA than did the corresponding zonal fraction of the adrenal gland of a previable foetus (experiment 5) incubated under the same conditions. The amounts of [^{3}H] DHA metabolized by the foetal zone fractions were similar in both experiments. The cells of the adult zone in the second half of pregnancy have been reported to contain more smooth endoplasmic reticulum and a more prominent Golgi complex than the cells of this zone of the adrenal glands of young foetuses (Johannisson, 1968). A development of the steroidogenic

capacity of the adult zone may, therefore, take place during the second half of gestation.

The conversions of [^{3}H] DHA to DHA sulphate in the adult and foetal zones were similar to those obtained with the corresponding zonal fractions of the previable foetus. Androstenedione formation was, however, much greater in both zonal tissues, especially the adult zone, than that observed at midterm. Villee and Loring (1964) reported that androstenedione was the major product of pregnenolone metabolism in homogenates of the adrenal glands of a newborn hydrocephalic infant whereas little conversion of pregnenolone to this steroid was obtained with adrenal tissue from previable foetuses. They concluded that the adrenal gland of the human foetus possesses much less 3β-hydroxysteroid dehydrogenase-isomerase activity than the adrenal glands of newborn infants. Also consistent with the presence of an active 3β-hydroxysteroid dehydrogenase-isomerase system in the adrenal gland of the neonate is the greater secretion of cortisol per square metre of body surface area by newborn infants than by older children or adults (Kenny, Malvaux and Migeon, 1963; Kenny, Preeyasombat and Migeon, 1966). There would appear therefore, to be a development of 3β-hydroxysteroid

dehydrogenase-isomerase activity in the adrenal gland during the second half of gestation or at birth. This might represent an adaptation of the foetus to meet the requirements of extrauterine life when Δ^4-3-oxosteroids are no longer supplied from the placenta.

Experiment 11 was the only instance in the present study of steroid formation from [^{3}H] DHA in a control incubation. The conversions to 11β-hydroxyandrostenedione in the whole tissue incubations were lower than in the control incubation. It is possible, therefore, that immersion of the reaction tubes containing tissue sections in a boiling water bath for 30 min failed to completely inactivate the enzymes present. Hall, Irby and Kretser (1969), however, reported the "conversion" of [^{3}H] cholesterol to testosterone in zero-time and heated enzyme controls in experiments with rat testicular tissue. The formation of 11β-hydroxyandrostenedione in the incubation with foetal zone tissue in the present experiment was of the same order as in the control incubation. The identification of this steroid as a metabolite of [^{3}H] DHA by the foteal zone of the adrenal gland of the newborn hydrocephalic infant must, therefore, be considered tentative. The

formation of 11β-hydroxyandrostenedione by adult zone tissue from this infant may indicate development of 11β-hydroxylase activity in the second half of pregnancy or at birth, or simply reflect the greater activity of 3β-hydroxysteroid dehydrogenase-isomerase present compared with midterm.

The higher conversions of [^{3}H] DHA to its sulphate by the whole adrenal gland preparations from the hydrocephalic infant than by either the adult or foetal zone fractions may be due to the presence in the whole tissue preparations of cells from the adult/foetal zone junction. High DHA sulphokinase activities were observed in cells from this region of the adrenal glands of the previable foetuses studied. Whole adrenal gland preparations from the hydrocephalic infant also possessed an active pregnenolone sulphokinase system (Cooke, 1970).

The metabolism of [^{3}H] DHA by adrenal tissue from newborn anencephalic infants.

In agreement with the results of other workers, adrenal tissue from the newborn infants investigated in the present study converted [^{3}H] DHA to DHA sulphate (Villee and Loring, 1969), androstenedione (Shahwan, Oakey and Stitch, 1968b; Villee and Loring, 1969) and 11β-hydroxyandrostenedione (Shahwan et al., 1968b). Johannisson (1968), while reporting some signs of cellular differentiation in the adrenal glands of anencephalic infants, considered the numerous vacuoles, poorly developed endoplasmic reticulum and mitochondria, and the absence of microvilli from the cells inconsistent with steroidogenic activity. Villee and Villee (1964) also suggested that the adrenal gland of the anencephalic infants is a relatively inactive tissue. The results of the present experiments, however, indicate extensive metabolism of [^{3}H] DHA by these glands in vitro. Adult zone tissue from the adrenal glands of the anencephalic infants used in the present study converted [^{3}H] pregnenolone to pregnenolone sulphate and 17α-hydroxypregnenolone, DHA and their sulphates (Cooke, Shirley and Taylor, 1970).

The more differentiated histological appearance of the adult zone of the adrenal gland of the post-mature anencephalic infant compared with this zone of the adrenal glands of the newborn hydrocephalic and premature anencephalic infants may reflect the greater maturity of this tissue. DHA sulphokinase, 3β-hydroxysteroid dehydrogenase-isomerase and 11β-hydroxylase activities were distributed throughout the adult zones of the post-mature anencephalic infant. Cameron, Jones, Jones, Anderson and Griffiths (1969) reported that DHA sulphokinase activity is restricted mainly to the zona reticularis of the adrenal gland of the human adult. Little differences in DHA sulphokinase and 3β-hydroxysteroid dehydrogenase activities in the different adult zones were apparent in the present study, although formation of androstenedione tended to decrease and of DHA sulphate to increase with increased depth in the gland. No striking differences in the metabolism of [^{3}H] pregnenolone were observed in the adult zones of the adrenal gland of the post-mature anencephalic infant by Cooke, Shirley and Taylor (1970). The metabolism of [^{3}H] DHA in the tissue

sections containing both inner dark and foetal zone cells suggests that, as in the adrenal glands of the newborn hydrocephalic infant and the previable foetuses, the foetal zone possessed greater DHA sulphokinase and less 3β-hydroxysteroid dehydrogenase activity than the adult zone.

The decreased conversions of [^{3}H] DHA to DHA sulphate in the zonal tissues of the adrenal gland of the post-mature anencephalic infant obtained in the absence of exogenous ATP were accompanied by increased formation of androstenedione in the outer dark, but not in the clear or inner dark zones. The increases in 3β-hydroxysteroid dehydrogenase-isomerase activity observed in the adrenal glands of previable foetuses, in the absence of extensive sulphoconjugation of DHA, were interpreted as indicative of a deficiency of this enzyme system relative to the levels of DHA sulphokinase present. The absence of markedly increased androstenedione formation in the adrenal gland of the post-mature anencephalic infant when ATP was omitted from the incubation medium might result from the higher 3β-hydroxysteroid dehydrogenase-isomerase activity present compared with the adrenal glands of the previable foetuses.

Much less [^{3}H] DHA was converted to 11β-hydroxyandrostenedione than was converted to androstenedione in all the zonal tissues investigated in the adrenal gland of the post-mature anencephalic infant. Similar results were obtained using slices of adrenal glands of newborn anencephalic infants by Shahwan et al., (1968b). 11β-hydroxyandrostenedione has been isolated in significant quantities from the adrenal glands of normal newborn infants (Matsumoto et al., 1968). Deshpande et al., (1970) have reported that hydroxylation of androstenedione represents a minor pathway of 11β-hydroxyandrostenedione synthesis in the adrenal gland of the human adult and that the major source of this steroid is cortisol. It is possible, therefore, that the principal route of 11β-hydroxyandrostenedione synthesis in the adrenal gland of the human foetus and newborn infant is from 11β-hydroxylated C_{21} steroids by a pathway excluding DHA and androstenedione as intermediates.

Villee and Loring (1969) have reported the conversion of [^{3}H] DHA sulphate to small quantities of 16α-hydroxyDHA by adrenal tissue from anencephalic infants. Virtually all the radioactivity incubated with adrenal tissue from the premature and post-mature anencephalic infants in the

present experiments was associated with carrier DHA, DHA sulphate, androstenedione and 11β-hydroxyandrostenedione. 16α-hydroxy DHA would not, therefore, appear to have been a quantitatively important metabolite of [^{3}H] DHA in these glands. No evidence for the formation of significant quantities of 16α-hydroxy DHA or its sulphate was obtained by radiochromatogram scanning of the incubation extracts in the experiments with previable human foetuses or the hydrocephalic infant. 16α-hydroxyDHA has been isolated from the adrenal glands of newborn infants (Matsumoto et al, 1968) and its sulphate from the adrenal glands of previable human foetuses (Huhtaniemi, Luukainen and Vihko, 1970). Shahwan, Oakey and Stitch (1969b) incubated [^{3}H] pregnenolone and [^{14}C] DHA with slices of adrenal glands from newborn anencephalic infants and isolated 16α-hydroxy DHA labelled with ^{3}H but not ^{14}C. The principal pathway of 16α-hydroxyDHA synthesis in the adrenal gland of the human foetus and newborn infant might, therefore, be from 16α-hydroxylated C_{21} steroids withcut the intermediate formation of DHA.

Urinary oestrogen excretion by women bearing an anencephalic foetus, while much greater than by the non-pregnancy female, is only about one-tenth of that observed

in normal pregnancies (Frandsen and Stakemann, 1961, 1964; Michie, 1966). DHA sulphate from the foetal adrenal gland is probably the quantitatively most important foetal precursor for placental oestrogen biosynthesis (Diczfalusy, 1967, 1969). Easterling, Simmer, Dignain, Frankland and Naftolin (1966) did not detect DHA sulphate in cord plasma in five anencephalic pregnancies although high levels of this steroid were present in cord blood during normal pregnancies. The urinary oestrogen excretion by the mother of the post-mature anencephalic infant used in the present study was 4 - 8 mg/24 h in weeks 36 - 41 of pregnancy - values typical of anencephaly. The efficient conversion of DHA to its sulphate by the adrenal gland of the foetus in vitro implies that the low oestrogen excretion was not a result of deficient adrenal DHA sulphokinase activity. Adrenal glands of anencephalic infants (including the post-mature infant used in the present study) converted much less [^{3}H] pregnenolone to DHA and DHA sulphate than the adrenal glands of previable foetuses (Cooke, 1970). These infants evidenced no lack of 17α-hydroxylase activity for pregnenolone as substrate. The small conversions to DHA and its sulphate appear,

therefore, to have been due to a deficiency of $C_{(17)}$-$C_{(20)}$ desmolase activity. This enzyme activity is low in the adult zone of previable foetuses and is confined mainly to the adult/foetal zone junction and the foetal zone of the foetal adrenal gland (Cooke, and Taylor, 1970). The low maternal urinary oestrogen excretion in anencephalic pregnancy would appear, therefore, to reflect a deficiency of $C_{(17)}$-$C_{(20)}$ desmolase, and not DHA sulphokinase, activity in the adrenal gland of the anencephalic foetus.

The adrenal gland of the premature anencephalic infant, like that of the post-mature anencephalic infant, possessed a high activity of DHA sulphokinase. This enzyme activity was very low in the adult zone of the adrenal glands of the previable foetuses used in the present study. If, as the histological studies of Meyer (1912) and Benirschke (1956) suggest, the adrenal gland of the anencephalic foetus develops normally during the first twenty weeks of gestation, a striking increase in DHA sulphokinase activity must occur in the adult zone during the second half of gestation or at birth. A development of this enzyme activity in the adult zone of the adrenal gland of the anencephalic foetus during the second half

of pregnancy might permit conversion of the limited amounts of DHA synthesised by these glands to DHA sulphate for placental oestrogen biosynthesis. This suggestion would be consistent with the low DHA sulphokinase activity detected in the adult zone of the adrenal gland of the hydrocephalic infant used in the present study.

Adult zone tissue from the adrenal gland of the premature anencephalic infant converted much less [^{3}H] DHA to androstenedione than the corresponding zone of the glands of the post-mature anencephalic and hydrocephalic infants. It is unknown if the premature anencephalic infant, which, unlike the other newborn infants studied, was delivered by Caesarean section, breathed before death. It is possible, therefore, as suggested by Bloch (1968), that adrenal 3β-hydroxysteroid dehydrogenase-isomerase activity is induced by changes in cellular environment effected by birth.

Shahwan, Oakey and Stitch (1968a) proposed that the adrenal cortex of the newborn anencephalic infant provides a useful source of adult zone tissue for biochemical investigation. The results obtained in the present study suggest, however, that the steroidogenic function of the adrenal gland of the anencephalic infant is different

from that of the adult zone of the adrenal glands of previable foetuses and normal newborn infants.

Concluding Remarks.

The metabolism of [^{3}H] DHA by adrenal tissue from previable human foetuses and a newborn hydrocephalic infant suggests that the foetal zone of the adrenal gland is an important source of the large amounts of sulphoconjugated Δ^5-3β-hydroxysteroids present in the blood, urine and tissues of the human foetus and neonate. The urinary excretion of 16α-hydroxylated Δ^5-3β-hydroxysteroids increases during the first five weeks of life, while the foetal zone is involuting (Reynolds, 1969). Reynolds (1969) has suggested that the precursors of these steroids are derived from the adult zone of the adrenal gland, which he proposed displays an underactivity of 3β-hydroxysteroid dehydrogenase-isomerase. This enzyme activity was, however, present in relatively high amounts in the adult zone of the adrenal gland of the newborn hydrocephalic infant. The observations of Reynolds may reflect an alteration of hepatic 16α-hydroxylase activity after birth when Δ^5-3β-hydroxysteroid sulphates produced by the foetal zone of the adrenal gland would no longer undergo placental aromatization.

Both histological zones of the human foetal adrenal gland were found to metabolize [^{3}H] DHA. The contribution

of the adult zone to DHA metabolism in the previable foetus would appear to be of very minor importance compared with that of the foetal zone and the adult/foetal zone junction. Evidence was obtained, however, for a development of the steroidogenic capacity of the adult zone, particularly with respect to 3β-hydroxysteroid dehydrogenase-isomerase activity during the second half of gestation or at birth. DHA sulphokinase activity was restricted to, in the main, the foetal zone and the adult/foetal zone junction of the adrenal glands of previable foetuses and the newborn hydrocephalic infant, while relatively high levels of this enzyme activity were present in the adult zone of the anencephalic infants studied. The histological abnormality of the adrenal glands of the anencephalic foetus appears to extend to abnormality of their steroidogenic function. The results of the present experiments, with the exception of those obtained with the adrenal gland of anencephalic infants, are consistent with the interpretations of Johannisson (1968) concerning the ultrastructure of the human foetal adrenal gland at different stages of gestation.

The findings of the present experiments emphasize the limitations of <u>in vitro</u> incubations with mixed cell populations

in the study of endocrine glands. The use of the microtechnique of Grunbaum *et al*, (1956), which provides a precise localization of the intraglandular site of cells used for incubation studies, enabled the detection of peaks of DHA sulphokinase and 3β-hydroxysteroid dehydrogenase-isomerase activity near the junction of the adult and foetal zones of the previable foetuses studied. It has also been shown that cells of similar histological appearance may display wide variations in their biochemical activities.

APPENDIX I

Histological staining of tissue sections.

Tissue sections obtained by the technique of Grunbaum, Geary & Glick (1956) were stained for histological examination with haematoxylin and eosin (H. & E.) or toluidine blue by the following procedures:

H. & E.

1.	5% (v/v) glacial acetic acid in ethanol	rinse
2.	distilled water	rinse
3.	Haematoxylin	2 min
4.	1% (v/v) HCl in ethanol	1 min
5.	0.17M-$MgSO_4$ containing 0.04M-$NaHCO_3$	30 sec
6.	distilled water	rinse
7.	1% (w/v) aq. Eosin	2 min
8.	distilled water	rinse
9.	30% aq. ethanol	rinse
10.	70% aq. ethanol	rinse
11.	ethanol	rinse
12.	xylene	rinse

Toluidine blue

1.	distilled water	rinse
2.	1% (w/v) aq. Toluidine blue	1 min

3.	distilled water	rinse
4.	90% aq. ethanol	rinse
5.	ethanol	rinse
6.	Xylene	rinse

Sections of whole adrenal tissue were prepared and stained with H. & E. by the staff of the Department of Pathology, Glasgow Royal Infirmary.

APPENDIX II

The estimation of protein nitrogen.

The protein nitrogen content of the tissue sections incubated with [^{3}H] DHA in the present study was estimated by a procedure similar to the Nayyar and Glick (1954) modification of the Greif (1950) bromsulphalein binding method.

Reagents.

Buffered bromsulphalein solution. 5% bromsulphalein (Hynson, Westcott and Dunning, Inc., Baltimore, Maryland, U.S.A.) (1 ml) was added to 1N-HCl (100 ml) and 1M-citric acid (50 ml) and made up to 250 ml with distilled water.

1.0N-NaOH

0.1N-NaOH

Procedure.

1. 4.0µl of 1.0N-NaOH was added to each to the protein precipitate in each reaction tube and the sample disintegrated by vibration mixing ("buzzing").

2. After 1 h at room temperature, buffered bromsulphalein solution (0.1 ml) was added and the contents of the tubes centrifuged at 1,500g for 5 min.

3. 0.1N-NaOH (1 ml) was added to 60 µl of each supernatant and the optical density measured at 580 mµ against a water blank.

4. Protein nitrogen content (µg) was calculated by subtracting the optical density of a reagent blank from that of each sample and multiplying the differences by 7.44.

The conversion factor (7.44) was obtained by parallel analysis of protein precipitates of foetal adrenal tissue by Kjeldahl nitrogen analysis (performed by Dr. A. Fleck of the Department of Pathological Biochemistry, Glasgow Royal Infirmary) and the above procedure. The use of this factor is permissible as long as the same batch of dye is employed. So little dye is required for each assay that the standardization procedure did not need to be repeated during the present study.

APPENDIX III

The chromatographic mobilities of steroids in the solvents systems used in the present study.

Steroid*	R_F value					
	I*	II	III	IV	V	VI
DHA	0.32	0.46	0.28		0.38	0.55
Δ^4A	0.45	0.55	-	-	0.52	0.74
11βΔ^4A	0.11	0.30	-	-	0.19	0.39
T	0.23	0.39	0.08	-	0.28	0.49
11βT	0.02	0.12	0.01	-	0.03	0.13
A'diol	0.14	0.31	-	-	0.20	0.34
16∝DHA	0.09	-	-	-	0.16	0.28
DHAAc	0.69	-	0.61	-	0.79	0.85
TAc	0.50	-	0.34	-	0.57	0.79
11βTAc	0.08	-	0.17	-	0.11	0.29
Neutral steroid monosulphates	-	-	-	0.4-0.5	-	-
Unconjugated steroids	-	-	-	0.9-1.0	-	-

* Abbreviations of steroids used in the present study are listed on p. xi .

* I Chloroform-acetone (37:3, v/v) ; II Cyclohexane-ethyl acetate (1:1,v/v) ; III Benzene-ethyl acetate (9:1, v/v);

/...

* IV tert.- Butanol-ethyl acetate-5N-NH_4OH (41:50:20, by vol.) ; V Chloroform-acetone (7:1, v/v); VI Chloroform-ethanol (19:1, v/v).

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