STEROID BIOGENESIS IN TESTICULAR TUMOURS

by

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II. Abbreviations used.

Trivial name.	Systematic name.
cholesterol:	cholest-5-en-3β-ol.
cortisol:	11β,17α,21-trihydroxypregn-4-ene-3,20-dione.
pregnenolone:	3β-hydroxypregn-5-en-20-one.
pregnenolone acetate:	3β-acetoxypregn-5-en-20-one.
11-oxopregnanetriol:	3α,17α,20α-trihydroxy-5β-pregnan-11-one.
17α-hydroxypregnenolone:	3β,17α-dihydroxypregn-5-en-20-one.
17α-hydroxypregnenolone acetate:	3β-acetoxy-17α-hydroxypregn-5-en-20-one.
16α-hydroxypregnenolone:	3β,16α-dihydroxypregn-5-en-20-one.
progesterone:	pregn-4-ene-3,20-dione.
17α-hydroxyprogesterone:	17α-hydroxypregn-4-ene-3,20-dione.

Trivial name.	Systematic name.
16α-hydroxyprogesterone:	16α-hydroxypregn-4-ene-3,20--dione.
DHA.	3β-hydroxyandrost-5-en-17-one.
DHA acetate:	3β-acetoxyandrost-5-en-17-one.
androst-5-ene-diol:	androst-5-ene-3β,17β-diol.
androst-5-ene-diol diacetate:	androst-5-ene-3β,17β--diacetate.
androst-4-ene-diol:	androst-4-ene-3β,17β-diol.
androstenedione:	androst-4-ene-3,17-dione.
testosterone:	17β-hydroxyandrost-4-en-3-one.
testosterone acetate:	17β-acetoxyandrost-4-en-3-one.
11β-hydroxyandrostenedione:	11β-hydroxyandrost-4-ene--3,17-dione.
adrenosterone:	androst-4-ene-3,11,17-trione.
11β-hydroxyandrosterone:	3α,11β-dihydroxy-5α-androstan--17-one.

Trivial name.	Systematic name.
11-oxotestosterone:	17β-hydroxyandrost-4-ene--3,11-dione.
11-oxotestosterone acetate:	17β-acetoxyandrost-4-ene--3,11-dione.
oestrone:	3-hydroxyoestra-1,3,5(10)--trien-17-one.
oestrone acetate:	3-acetoxyoestra-1,3,5(10)--trien-17-one.
oestrone-3-methyl ether:	3-methoxyoestra-1,3,5(10)--trien-17-one.
oestradiol-17β:	oestra-1,3,5(10)-triene--3,17β-diol.
oestradiol-17β-diacetate:	oestra-1,3,5(10)-triene--3,17β-diacetate.
oestradiol-17β-3--methyl ether:	3-methoxyoestra-1,3,5(10)--trien-17β-ol.
oestradiol-17α:	oestra-1,3,5(10)-triene--3,17α-diol.

Trivial name.	Systematic name.
oestradiol-17α-diacetate:	oestra-1,3,5(10)-triene--3,17α-diacetate.
oestriol:	oestra-1,3,5(10)-triene--3,16α,17β-triol.
oestriol triacetate:	oestra-1,3,5(10)-triene--3,16α,17β-triacetate.
oestriol-3-methyl ether:	3-methoxyoestra-1,3,5(10)--triene-16α,17β-diol.
equilenin:	3-hydroxyoestra-1,3,5(10),-6,8(9)-pentaen-17-one.
diethylstilboestrol:	3,4-bis(p-hydroxyphenyl)--3-hexene.
cholesterol sulphate:	cholest-5-ene-3β-sulphate.
pregnenolone sulphate:	3β-sulphoxypregn-5-en--20-one.
17α-hydroxypregnenolone sulphate:	3β-sulphoxy-17α-hydroxypregn--5-en-20-one.
16α-hydroxypregnenolone sulphate:	3β-sulphoxy-16α-hydroxypregn--5-en-20-one.

Trivial name.	Systematic name.
16α,17α-dihydroxypregnenolone sulphate:	3β-sulphoxy-16α,17α-dihydroxypregn-5-en-20-one.
DHA sulphate:	3β-sulphoxyandrost-5-en--17-one.
16α-hydroxy-DHA sulphate:	3β-sulphoxy-16α-hydroxyandrost-5-en-17-one.
androst-4-ene-diol sulphate:	one or more of the sulphated forms of androst-4-ene-diol.
androst-5-ene-diol sulphate:	one or more of the sulphated forms of androst-5-ene-diol.
androst-5-ene-diol-3--sulphate:	3β-sulphoxyandrost-5-en--17β-ol.
testosterone sulphate:	17β-sulphoxyandrost-4-en--3-one.
oestrone sulphate:	3-sulphoxyoestra-1,3,5(10)--trien-17-one.
oestriol-3-sulphate:	3-sulphoxyoestra-1,3,5(10)--triene-16α,17β-diol.

Trivial name.	Systematic name.
I.C.S.H.:	Interstitial cell stimulating hormone.
F.S.H.:	Follicle stimulating hormone.
A.C.T.H.:	Adrenocorticotrophic hormone.
Testicular Tumour Panel:	Testicular Tumour Panel and Registry of the Pathological Society of Great Britain and Ireland in association with the British Empire Cancer Campaign for Research.
ATP:	adenosine 5'-triphosphate.
NADP:	nicotinamide-adenine dinucleotide phosphate.
dpm:	disintegrations per minute.
E.C.:	Enzyme Commission number (see "Enzyme Nomenclature" Elsevier Publ. Co., Amsterdam, 1965).

III. Introduction.

Tumours arising in mammalian endocrine tissues provide the endocrinologist with material that may help to throw further light on the metabolism of the cancer cell and on the enzymic activity of a particular cell type which would otherwise prove very difficult to isolate in the pure state owing to the particularly close association that dissimilar cells often have with each other. Such is the case with Sertoli cells, the spermatogenic epithelial elements and Leydig cells in the mammalian testis. In addition, if a particular tumour arises in more than one species of animal a unique comparative study is thus made available.

Such a situation presents itself in the case of testicular tumours occurring in man and dog where neoplasms of one particular cell type do occur and are histologically so similar in the two species as to provide difficulty in their differentiation.

Certain of these testicular tumours are associated with obvious clinical signs of hormonal imbalance in the host indicating a probable derangement of testicular

enzyme systems. It was thus considered a worthy project to investigate the steroidogenic ability of those tumours of the testis that were associated with, in particular, feminization in man and dog.

An outline is presented below of the knowledge accrued since early times of testicular function in relation to its endocrine status and of the pertinent data on testicular tumours in man and dog where signs of hormonal dysfunction are clinically manifest.

The testis has been associated with "maleness" since ancient times as indicated by the castration of men (St. Matthew, Ch.19, v.12) and his domestic animals (Aristotle, c. 344 B.C., a) and the changes observed in appearance and behaviour thereafter (Aristotle, c. 344 B.C., b).

John Hunter (1794) was probably the first to demonstrate experimentally the hormonal activity of the testis by grafting a rudimentary spur from a hen on to the leg of a cock and noting its development into a strong masculine appendage. The small spur from a young cock, however, underwent a very much slower development when implanted into a hen's leg. Berthold (1849) showed that comb-shrinkage following

castration could be counteracted by testicular transplantation into other parts of the body. Other workers (Loewy, 1903; Walker, 1908,a,b; Pezard, 1911) have also demonstrated the masculinizing properties of testicular extracts in poultry.

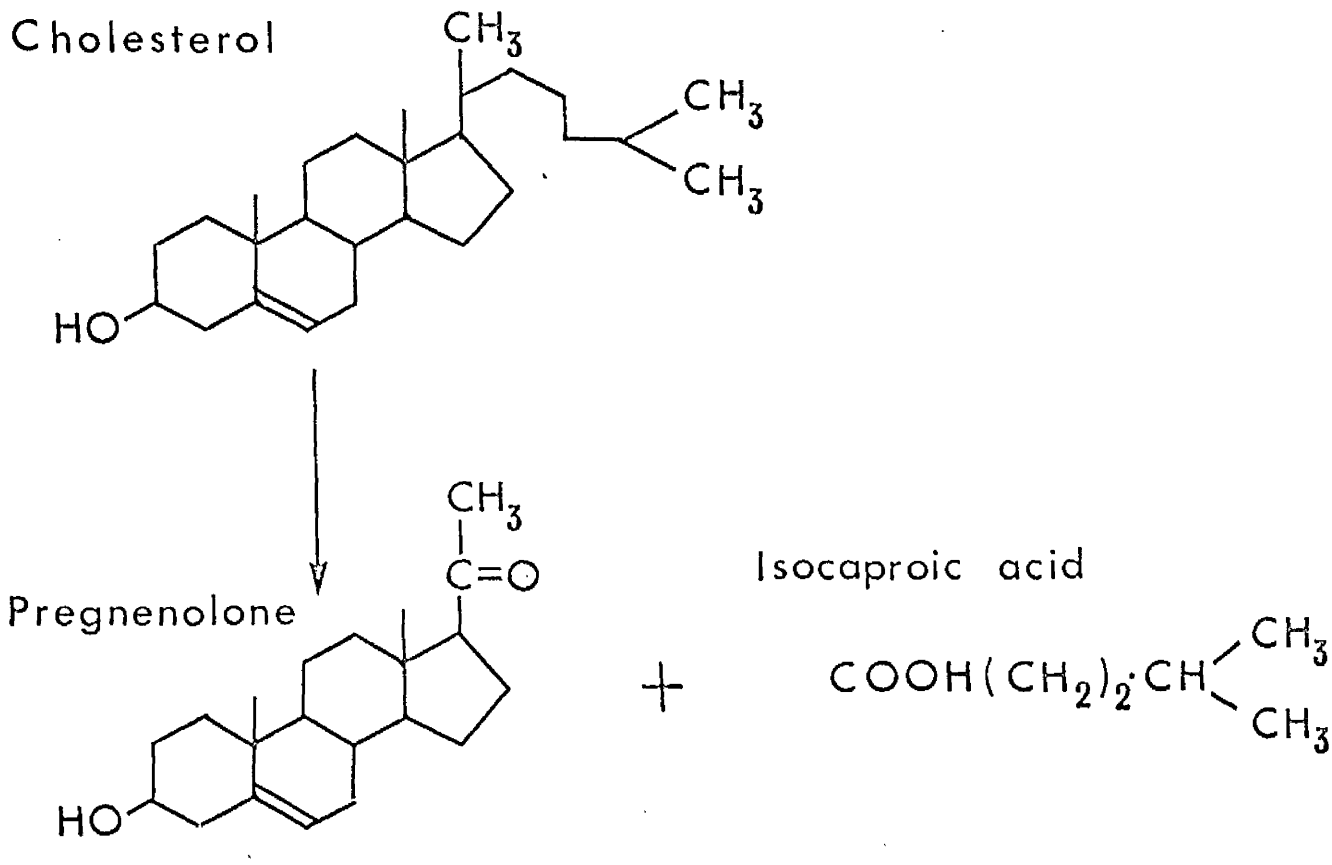
The isolation of an androgenic substance from bulls' testes (McGee, 1927) started the investigations into the chemical nature of testicular hormones and the process of their biosynthesis. Testosterone was identified by David, Dingemanse, Freud & Laqueur (1935) while evidence was provided by the early perfusion studies of Danby (1937, 1938, 1940) that this was the main androgen secreted by the testis of the bull. She postulated that it could be formed from DHA, androstenedione and androst-5-ene-diol. Testosterone was eventually shown to be a secretory product of the testis of the dog (West, Hollander, Kritchevsky & Dobriner, 1952) and of man (Lucas, Whitmore & West, 1957).

The idea that cholesterol might be a precursor of the steroid hormones developed as the chemical structures of the hormones isolated from the steroid producing glands were found to be perhydrocyclopentenophenanthrene derivatives bearing an obvious chemical relationship to

cholesterol. The work of Werbin & LeRoy (1954), Zaffaroni, Hechter & Pincus (1951), Werbin, Plotz, LeRoy & Davis (1957), Ungar & Dorfman (1953), Bloch (1945) and others has shown that cholesterol is a common precursor of virtually all of the known steroid hormones. Enzymic cleavage of its side-chain yields isocaproic acid and pregnenolone (Staple, Lynn & Gurin, 1956) (Text-fig. 1). Pregnenolone had previously been isolated from testicular tissue by Ruzicka & Prelog (1943).

The advent of isotopically-labelled steroids in the 1950's allowed for more intimate investigations into the biosynthesis of testicular hormones. Carbon-labelled acetate was shown to be converted to cholesterol when incubated with rat testicular tissue <u>in vitro</u> (Srere, Chaikoff, Treitman & Burstein, 1950). Brady in the following year reported the ability of this same tissue to synthesize ~~4-~~^{14}C testosterone from ~~4-~~^{14}C acetate and suggested that cholesterol may not be an obligatory precursor of testosterone. Hall (1963, 1966), on the other hand, has provided evidence that cholesterol is an intermediate in the biosynthesis of testosterone by rabbit testicular tissue and that interstitial cell stimulating hormone

Text-figure 1.

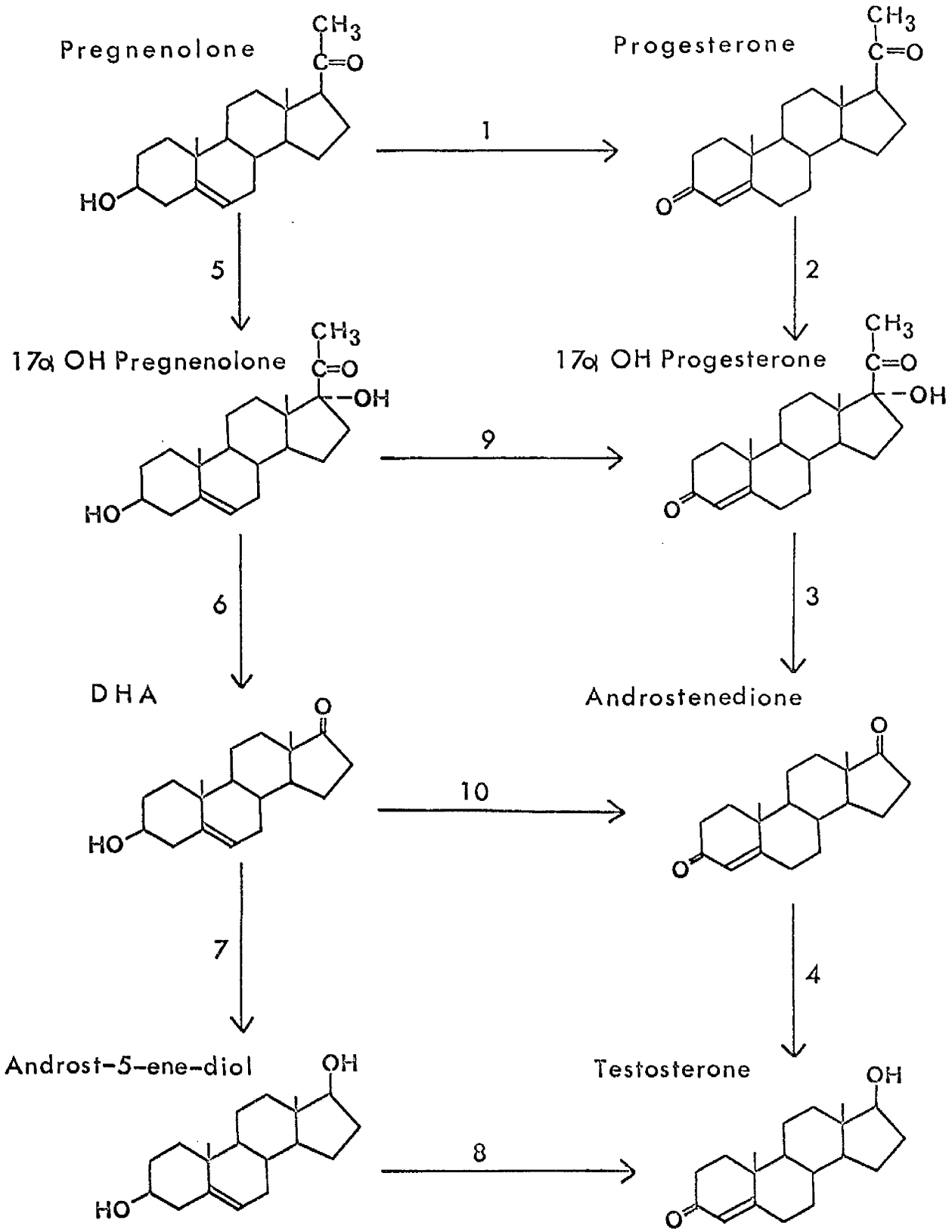


acts between cholesterol and pregnenolone formation.

The enzyme system 3β-hydroxysteroid dehydrogenase--isomerase, necessary to convert pregnenolone to progesterone (Text-fig. 2, reaction 1) has been demonstrated in rat testicular tissue by Samuels, Helmreich, Lasater & Reich (1951). The further conversion of this compound was shown to be possible by the enzyme studies of steroid oxidations (Lynn & Brown, 1956). These authors showed the presence of 17α-hydroxylase, 17α-hydroxypregnene C_{17}-C_{20}lyase and 17β-hydroxysteroid dehydrogenase enzymes in rat testicular particles. Slaunwhite & Samuels (1956) formulated a biosynthetic pathway for the production of testosterone by testicular tissue. Progesterone is converted to 17α-hydroxyprogesterone (reaction 2), side-chain cleavage forms androstenedione (reaction 3) and then the reduction of this compound to testosterone. A second pathway was proposed by Kahnt, Neher, Schmid & Wettstein (1961) after the suggestion by Glen & Heftman (1951) and Savard & Dorfman (1954) that DHA might be an intermediate in the synthesis of testosterone. They postulated that pregnenolone was converted to 17α-hydroxypregnenolone (reaction 5), and the side-chain

Text-figure 2.

BIOSYNTHESIS OF ANDROGENS IN THE CANINE TESTIS.



was then removed to form DHA which is then converted to androstenedione (reactions 6 & 10) and finally to testosterone by reaction 4.

The existence of these pathways has been substantiated by the in vivo and in vitro studies of Eik-Nes and his co-workers. Eik-Nes & Kekre (1963) suggested that the conversion of pregnenolone to 17α-hydroxypregnenolone might be more important in the dog testis than the formation of progesterone, a steroid hormone, from pregnenolone, and then use the route from 17α-hydroxypregnenolone to 17α-hydroxyprogesterone (reaction 9) both of which steroids have low biological activities. Hagen & Eik-Nes (1963, 1964a) reported the conversion of 17α-hydroxypregnenolone to 17α--hydroxyprogesterone with the formation also of DHA, androstenedione and testosterone. These same authors (1964b) showed that DHA was metabolized at a faster rate than 17α-hydroxyprogesterone to androstenedione and suggested that the latter steroid was not an obligatory intermediate in the formation of testosterone. Rosner, Hosita & Forscham (1964) suggested androst-5--ene-diol as an intermediate. This steroid has been shown to be converted to testosterone by placental and

adrenal tissue (Baulieu, Wallace & Lieberman, 1963) and recently by Slaunwhite & Burgett (1965) using rat testicular homogenates. These authors suggested that DHA is not an important intermediate in testosterone biosynthesis being rapidly converted to androstenedione which, in this particular preparation, does not rapidly form testosterone. Equilibrium between androstenedione and testosterone was not achieved in this tissue even after 3 hr. incubation and at that time the former was found to be present in greater amounts. They also suggested that 17α-hydroxypregnenolone may be converted to androst-5-ene-diol without the intermediation of DHA.

Fevold and Eik-Nes (1963) have demonstrated the presence of 20α and 20β hydroxysteroid-dehydrogenases in testicular tissue and were able to isolate the 20α and 20β reduction products of progesterone and 17α--hydroxyprogesterone from their incubations with testicular tissue. Ellis & Berliner (1965) also reported the isolation of 17α,20α-dihydroxypregn-4--en-3-one and the transformation of this steroid into testosterone.

The studies of Kase, Forchielli & Dorfman (quoted by Dorfman, 1963) indicate a pathway from progesterone

to testosterone via testosterone acetate. These authors found that the incubation of 7α-^{3}H progesterone and 4-^{14}C 17α-hydroxyprogesterone with a homogenate prepared from human polycystic ovaries yielded testosterone containing a higher ratio of $^{3}H/^{14}C$ than that found for the androstenedione simultaneously formed. This strongly suggests that testosterone could be formed from progesterone without the intermediation of either 17α-hydroxyprogesterone or androstenedione. Fonken, Murray & Reineke (1960) established this supposition by isolating 21-^{14}C testosterone acetate during the transformation of 21-^{14}C progesterone to testosterone by Cladosporium resinae. Had the pathway involved 17α-hydroxylation of progesterone and side-chain splitting the testosterone acetate isolated would not have borne the ^{14}C-label. Testosterone incubated with the microörganism did not give testosterone acetate. Forchielli, Gut & Dorfman (1961) described the direct conversion of progesterone to testosterone acetate by the formation of an epoxy bridge between carbons 17 and 20 of progesterone and the simultaneous rupture of the direct linkage between these two carbons. To examine the possibility that

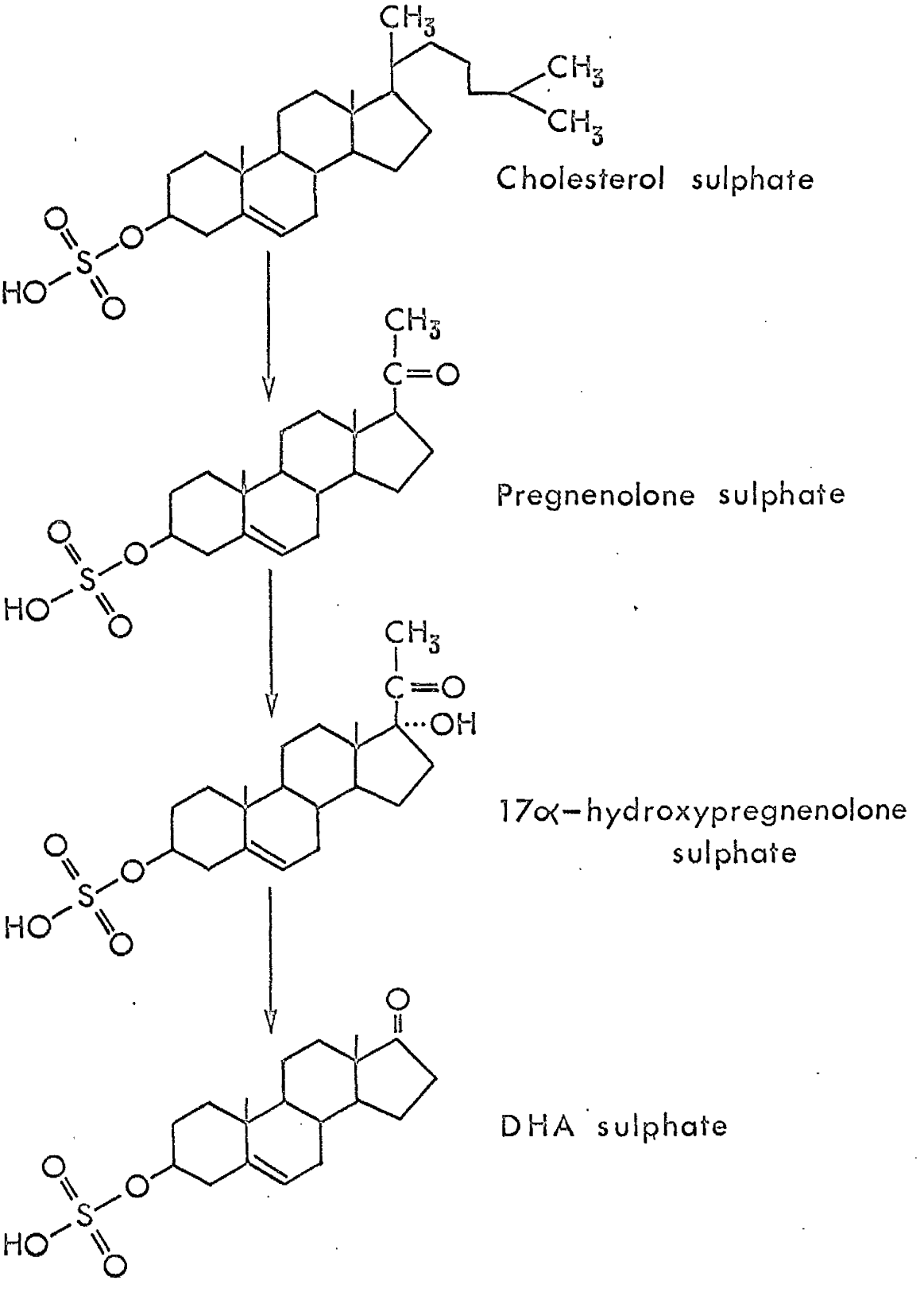
17α-hydroxyprogesterone might not be an obligatory intermediate in the formation of testosterone from progesterone, Forchielli et al. (1961) incubated progesterone containing tritium in the 17α-position with homogenates of rat testes and found that the testosterone isolated was in fact tritiated. The isotope was in the 17α-position to indicate, by its disappearance or otherwise from the steroid molecule, whether oxidation to androstenedione had occurred prior to testosterone formation.

The demonstration of sulphation of steroids in endocrine tissues (Baulieu, 1962; Sneddon & Marrian, 1963; Wallace & Lieberman, 1963), suggested the possibility of new pathways for steroid hormone biosynthesis via these conjugates. That biosynthesis could proceed via the intact steroid sulphates was demonstrated by Calvin, VandeWiele & Lieberman (1963), Calvin & Lieberman (1963), and Roberts, Bandi, Calvin, Drucker & Lieberman (1964a,b) (Text-fig. 3). It has been demonstrated moreover, that the dog testis is able to convert DHA sulphate to testosterone and androstenedione in vivo (Aakvaag, Hagen & Eik-Nes, 1963).

Calvin et al. (1963) have pointed out that the regulatory processes determining end-product formation and their amounts are, as yet, not understood. The

Text-figure 3.

Steroid sulphates as biosynthetic intermediates.



enzyme systems responsible for the various conversions indicated above are intimately involved with this control. Derangement of any one, or more, of these systems may provide for the non-production of a particular intermediate or in the formation of an abnormal hormone and the development of clinical syndromes.

The 3β-sulphoxysteroids isolated provide for further possibilities for consideration as loci where derangement can occur.

These steroids and their enzymes will be discussed further in the light of the findings from the investigations of steroid biosynthesis in testicular tumours presented herein.

The question has inevitably arisen as to which of the testicular cells are responsible for the synthesis and secretion of these steroid hormones. Histologically the testis may be divided into seminiferous tubules and the interstitial cells of Leydig. The latter are disposed in a connective tissue stroma between the tubules and are permeated by a capillary network. Ancel & Bouin (1903a, 1904a,b) appear to have been among the first to investigate the problem of the site of production of the testicular hormone. They observed

in horses, dogs and pigs the degeneration of seminal epithelium of retained testes without immediate atrophy of the accessory gential organs and without degeneration of the interstitial tissue. Ligation of the efferent ducts caused complete disappearance of the seminal epithelium without the animals losing their masculinity. The work suggested that the interstitial cells were responsible for male hormone production. Moore (1932) recorded that an abdominal testis was secreting twice as much androgen as a normal testis and it contained no seminiferous cells. Moore & Samuels (1931) working on the converse showed involution of the prostate and seminal vesicles when atrophy of the interstitium had been induced by a deficient diet. The excretion of 17-oxosteroids and the development and condition of secondary sex characteristics parallel histologic and cytologic evidence of secretory activity by the interstitial cells (Albert, Underdahl, Greene & Lorenz, 1955) .

Exposure of the testes to x-rays was noted, at the turn of the century, to produce sterility, without impairing androgenic function. Tandler & Grosz (1912) observed that an irradiated roe-deer became infertile

but cast its antlers and subsequently regenerated them in the normal way. Nemenov (1916) destroyed the germinal epithelium of a dog without affecting the development of the prostate. A more quantitative investigation by Abbott (1959) showed a very rapid drop in weight of the testes of rats exposed to high doses of x-rays which was associated with complete destruction of the seminal line but with normal intertubular tissue and accessory organs. Copulatory activity remained normal.

Many other types of destructive procedures have been used to remove a particular cellular element including so-called "Cadmium-castration". Parizek (1960) showed permanent destruction of the Sertoli and germinal cells but the Leydig cells eventually recovered along with a rise in androgenic activity. This regeneration has been observed biochemically (Favino, Baillie & Griffiths, 1966).

Spontaneous failure of the seminiferous tubules has been recorded as in the syndrome described by Klinefelter, Reifenstein & Albright (1942) in which the patient presents with small testes containing hyalinised tubules but abundant Leydig cells, azoospermia and high

gonadotrophin excretion. Heller & Nelson (1945) stressed the histological normality of the Leydig cells, the frequent association of complete tubular failure and yet normal masculinity of the men.

Histochemical techniques (Kunze, 1922; Pollock, 1942) applied to investigations of the distribution of testis lipids have revealed, in almost every instance studied, that the testis cholesterol is localized predominantly in the interstitial cells. Pollock (1942) suggested a relationship between the location of "ketosteroids" in the interstitial cells to testosterone content. Pearlman (1950), on the other hand, found no apparent relationship between changes in testis cholesterol and interstitial cell function, but did suggest that the cholesterol might be localized predominantly in the seminiferous tubules.

Huggins & Moulton (1948) found a linear correspondence between the esterase activity of the Leydig cells and their androgen production. Wattenberg (1958) localized the 3β-hydroxysteroid dehydrogenase enzyme only in the interstitial cells of Leydig after he had incubated testis slices from the albino rat with pregnenolone and DHA. This latter work has since been

confirmed many times (Baillie, Niemi & Ikonen, 1965; Baillie & Griffiths, 1964; Niemi & Ikonen, 1963; Steinberger, Steinberger & Vilar, 1966). Niemi & Ikonen (1963) related the esterolytic activity with the activity level and distribution of the 3β-hydroxy-steroid dehydrogenase. On a purely histochemical basis Niemi, Harkönen & Ikonen (1966) showed that the esterase (hydrolase) activity of the Sertoli cells differs markedly from that of the interstitial cells. The former, an acetic-ester hydrolase (E.C. 3.1.1.6) has a substrate preference for indoxyl acetate rather than naphthyl acetates. The esterase activity of the interstitial tissue falls into the acetic-ester hydrolase type (E.C. 3.1.1.6). Hypophysectomy results in a rapid decrease of some oxidative enzyme activity including that of 3β-hydroxysteroid dehydrogenase of the interstitial tissue (Niemi & Ikonen, 1962), whereas the two types of nonspecific esterase activity responded quite differently (Niemi et al., 1966). The acetic--ester hydrolase activity in the interstitial cells decreased rapidly after cessation of the gonadotrophin stimulation, whereas the acetic-ester hydrolase activity occurring in both the Sertoli and interstitial cells

increased. On the basis of their results Niemi et al. (1966) concluded that there are two functionally different categories of esterases (hydrolases) in the testis tissue; one sensitive to gonadotrophin stimulation which can be demonstrated chemically with p-nitrophenyl propionate as substrate and which is related to steroid biosynthesis and another non-specific hydrolase which utilizes β-naphthyl acetate and which is not connected with the endocrine function of the testis and hence is not affected by hypophysectomy. This latter fraction is dependant upon the amount of the interstitial connective tissue and Sertoli cells but independant of the number or functional state of the Leydig cells or germ cells.

Christensen & Mason (1965) isolated Leydig cells and seminiferous tubules from rat testis and incubated each with 4-^{14}C progesterone. They found that both tissues were capable of transforming this steroid to 17α-hydroxyprogesterone, androstenedione and testosterone although the interstitial tissue was considerably the more active. This work confirmed the histochemical findings cited above that the interstitial cells are the principle site of androgen production in the testis

but showed also the ability of the seminiferous tubules to transform steroids and synthesize androgens in vitro probably to the extent of 10% of the total androgen production of the testis.

Oestrogenic activity of testicular extracts was shown as early as 1921 by Fellner and shortly afterwards in male urine (Lacqueur, Dingemanse, Hart & de Jongh, 1927). The phenomenally high levels of oestrogenic activity in stallions' urine was shown initially by Küst (1932) and two years later oestrone was isolated from this source (Häussler, 1934; Deulofeu & Ferrari, 1934). The involvement of the testis in the production of these oestrogens was again emphasised by Zondek (1934) who demonstrated a far greater activity in the urine from stallions than that from geldings. The actual isolation of oestrone and oestradiol-17β in crystalline form from horses' testes was achieved by Beall (1940).

Direct evidence for the testicular formation of oestrogens was demonstrated using homogenates in the horse (Baggett, Engel, Balderas & Lanman, 1959) and by Nyman, Geiger & Goldzieher (1959) who perfused testes with acetate-1-^{14}C and showed the production

of radioactive oestrone and oestradiol-17β.

The site of origin of oestrogens in the testis is still in dispute. A tubular origin has been suggested by the occurrence of oestrogens in semen (Diczfalusy, 1955) whilst preparations of human semen were shown to cause an enlargement of the uterus when injected into immature rats (Green-Armytage, Silberstein & Wachtel, 1947).

The production of a hormone, distinct from the androgens, by the germinative epithelium was suggested by the parabiotic experiments of Martins & Rocha (1931). McCullagh (1932) designated this hormone "inhibin" and showed it to have pituitary-inhibiting properties quite distinct from the androgens. Klinefelter et al. (1942) pronounced a "dual hormone theory of testis physiology" there being a second hormone from the germinal epithelium which is very similar to an oestrogen. Tornblöm (1942) demonstrated that in castrated rats testosterone would cause growth of the prostate yet not prevent pituitary hypertrophy. Howard, Sniffen & Simmons (1948) proposed that a gonadotrophin, presumably equivalent to FSH, stimulates the Sertoli cells which secrete a hormone

that provokes spermatogenesis, inhibits the production of FSH and probably influences the secretion of ICSH. Lacy (1962) whilst working on the regulation of the spermatogenic cycle in the mammalian testis described a metabolic cycle of the Sertoli cells which was dependant upon spermatogenesis and synchronised with it. He studied by light and electron microscopy the phagocytosis of "residual bodies", first described by Regaud (1901), by the Sertoli cells followed by an increase in their lipid content. This lipid gradually disappeared during the next five stages of the epithelial cycle, becoming minimal at stage fourteen (Leblond & Clermont, 1952). Lacy (1962) considered this disappearance to be indicative of hormone-release which influenced spermatogenesis. He suggested that the "residual bodies" initiate or accelerate steroid synthesis by the Sertoli cells. The Sertoli cell hormone (SCH) is then utilized during spermatogenesis and its synthesis and/or release is dependant upon FSH. That spermatogenesis requires androgens has been shown (Boccabella, 1963) and that the seminiferous tubules are capable of their synthesis (Christensen & Mason, 1965) may provide for tubular function

independant of the biosynthetic products of the Leydig cells. Christensen & Mason (1965) were unable to demonstrate the formation of either oestrone or oestradiol-17β from progesterone, testosterone or androstenedione during incubation studies with separated seminiferous tubules and Leydig cells from rat testes.

Leach, Maddock, Tokuyama, Paulsen & Nelson (1956) maintain, on the other hand, that oestrogen excretion is a better index of Leydig cell function than is androgen excretion. Maddock & Nelson (1952) administered chorionic gonadotrophin to men and found a two-fold increase in urinary 17-oxosteroids as compared with an elevation of oestrogen five to sixteen--fold in conjunction with hyperplasia of the Leydig cells.

Electron micrograph studies of testicular cells have produced equivocal results with regard to morphological correlation with endocrine function. Christensen & Fawcett (1961, 1966) and Christensen (1963) postulate that the abundant agranular endoplasmic reticulum of the Leydig cells of the opossum, mouse and guinea pig testis respectively indicates that this is the site of at least part of steroid

hormone production in the light of the findings of Lynn & Brown (1958) that the microsomal fraction of guinea pig testis homogenates contained at least two ribonuclease-stable enzymes involved in androgen biosynthesis. The enzymes for the metabolism of progesterone in the mouse testis are found mainly in the microsome fraction (Murota, Shikita & Tamaoki, 1965). It is agreed that the microsome fraction derives predominantly from the endoplasmic reticulum of intact cells and thus Christensen & Fawcett (1966) conclude that the enzymes necessary to transform progesterone to 17α-hydroxyprogesterone, androstenedione and finally testosterone are presumably associated with the abundant agranular endoplasmic reticulum of mouse interstitial cells. The mitochondria of these cells contain the tubular cristae that are typical of mitochondria in many steroid-secreting cells (Belt & Pease, 1956).

Brökelmann (1963), on the other hand, demonstrated that the Sertoli cells of the rat testis also contain an agranular endoplasmic reticulum, mitochondria with tubular cristae, lipid droplets and/or inclusion bodies resembling lysosomes, which picture, he suggests,

fits in well with the cytoplasmic morphology of steroid-producing cells.

Signs of abnormal hormonal production are often associated with testicular tumours in man and dog and have been hailed by many investigators as indicating the steroidogenic ability of the parent cell, with some equivocy of interpretation.

Neoplasia of the testis in man is rare, occurring at a rate of 2.3 per 100,000 males (Collins & Pugh, 1964) with an annual incidence of 550 to 600 new cases in the United Kingdom. Dixon & Moore (1952) attributed 0.64% of all male cancer deaths in the United States of America to this cause, while the comparable rate for this country in 1938-39 was 0.52% (Harnett, 1952). Signs of hormonal imbalance have frequently been found in conjunction with interstitial tumours of the testis in man. The possible dual role that the Leydig cell may play in normal steroid synthesis is indicated by the two diametrically opposite forms of endocrine disorder with which tumours of this cell type are associated. Its occurrence is rare; 1.4% of the testicular tumours examined by the Testicular Tumour Panel

Table 1. The relative incidence of some human testicular tumours and the frequency of malignancy and associated endocrine effects.

(Collins & Pugh, 1964)

Type	No.	% of total	Endocrine changes		Malignant	
			No.	% of group	No.	% of group
Seminoma	400	40	5	1.3	55	13.7
Teratoma	322	32	13	4.0	156	49
Combined teratoma and seminoma	136	14	6	4.4	57	42
Sertoli cell tumour	6	0.6	1	16.7	1	16.7
Interstitial cell tumour	14	1.4	6	43	1*	7.1
Orchioblastoma	8	0.8	-	-	3	38
Malignant lymphoma	66	6.6	-	-	41	62

* not proven.

(Collins & Cameron, 1964) were of this type and 1.2% in the series of Dixon & Moore (1952). The Panel reported 43% of this group to be associated with gynaecomastia in adults, indicating feminizing tendencies (Table 1.). This same growth occurring in the juvenile testis would appear to be almost invariably associated with precocious sexual development. The children generally present with macrogenitosomia, pubic hair, deepening of the voice, precocious muscular and skeletal development and psychosexual precocity. Acne is sometimes noted. Bishop, Van Meurs, Willcox & Arnold (1960) reported such a case in an 11 yr. old boy and reviewed 25 further cases from the literature. Levels of urinary 17-oxosteroids in those cases where estimations were recorded, were found to be elevated. The sexual precocity has been ascribed to "associated congenital adrenal hyperplasia" (Abelson, Bulaschenko, Trommer & Valdes-Dapena, 1966). These authors suggest that probably the tumour itself results from ACTH stimulation. In a few instances testicular tumours in children have been associated with Cushing's syndrome (Hamwi, Gwinup, Mostow & Besch, 1963; Crouch, 1958; Engel, McPherson, Fetter & six others, 1964).

Endocrine features, if any, arising in the adult in association with the interstitial cell tumour are gynaecomastia, decreased libido and impotence. It has been suggested that androgenic manifestations, as occur in children with this type of neoplasm, cannot occur in the adult as full virilization is already present. It is not clear whether the mammary hypertrophy is due to tumour oestrogens, testicular androgen stimulated by tumour gonadotrophins (Gabrilove, Sharma, Wotiz & Dorfman, 1965) or tumour androgens acting directly on the breast. In the case described by Abelson et al. (1966) the moderate rise in urinary oestrogens was not associated with gynaecomastia. The levels of urinary 17-oxosteroids were, however, abnormally high and fractionation revealed that both 11-oxygenated and 11-deoxy steroids were increased. The presence of 11β-hydroxylase, considered previously to be a specific enzyme of the adrenal cortex, has been described (Savard et al., 1960; Dominguez, 1961; Smith, Breuer & Schriefers, 1964) in testicular tumour tissue. Besch, Watson, Barry, Hamwi, Mostow & Gwinup (1963) considered the testicular tumour they investigated, which was shown to synthesize

cortisol, was of adrenocortical origin.

Sertoli cell tumours of the human testis occur less frequently than in the dog; 6 out of 995 testicular tumours were so described by the Testicular Tumour Panel (Collins & Symington, 1964) (Table 1). One patient, aged 27 yr., also had enlargement of the breasts. Gynaecomastia has been reported previously to be associated with this tumour (Ostergaard, 1947; Teilum, 1949, 1958; Jones & Friedman, 1950; Lewis & Stockard, 1950; Fuglsand & Ohlsen, 1957).

The occurrence of testicular tumours in the dog is high. Dow (1962) found 16% of 580 unselected dogs examined at the Glasgow University Veterinary Hospital to be so affected, whilst Cotchin (1954) reported 5.8% of 2,361 canine tumours to be of the male gonad. It is of interest that all testicular tumours in the dog are of Sertoli, interstitial or spermatogonial cell origin. The absence of teratomas has been the striking feature of all published surveys of canine testicular tumours. The relative incidence, respectively, of these three types of tumour in the dog is given as 39%, 60% and 48% by Dow (1962) and 49%, 17% and 34% by Cotchin (1960).

Table 2. The relative incidence of canine testicular tumours and the frequency of malignancy and associated endocrine effects.

Type	No.	% of total	Endocrine changes		Malignant	
			No.	% of group	No.	% of group
Dow (1962)						
Sertoli cell tumour	36	39	6	17	5	14
Interstitial cell tumour	56	60	0	0	1	1.8
Seminoma	45	48	0	0	4	9
Cotchin (1960)						
Sertoli cell tumour	157	50	45	29	2	1.3
Interstitial cell tumour	54	17	16‡	30	1	1.9
Seminoma	107	34	4⁂	3.8	2	1.9

‡ only 4 out of the 16 reliable.

⁂ no definite evidence for any of these 4.

Both authors found bilateral and multiple tumour involvement of testes to be high. Table 2 summarizes their findings, and Table 1 indicates the relative incidence of some human testicular tumours and the frequency of malignancy and endocrine effects.

The frequency with which clinical signs of hormonal imbalance are associated with testicular tumours in dogs is an outstanding feature of testicular cancer. The canine Sertoli cell tumour has attracted particular attention in this respect and has been used in evidence for the hypothesis that the parent cell is the site of elaboration of oestrogens in the normal gland. The changes that occur in those dogs thus affected have been ~~eluded~~ alluded to many times (Greulich & Burford, 1936; Zuckerman & Groome, 1937; Zuckerman & McKeown, 1938; Innes, 1942). The feminization is manifested by enlargement of the teats, with or without mammary hypertrophy, and attractiveness to other male dogs. The glands may secrete a serous or milky fluid in extreme cases. The posture adopted for micturition may change from the three-legged stance of the male to the squatting position of the bitch. The animal is generally

presented for professional advice when the owner observes abnormal skin and coat changes which are common indications of the syndrome. The hair begins to fall out from the region of the ventral thorax and abdomen, the posterior and lateral aspects of the thighs and occasionally where there is friction as in the neck and shoulder area. The distribution of hair loss may become quite widespread and is bilaterally symmetrical. The skin is usually thin and velvetty in texture but may, on the other hand, be thick and tough, and show, on histological examination, a great increase in collagenous fibres characteristic of scleroderma (Brodey & Martin, 1958). A diffuse, dusky, purple pigmentation is also noted in these areas. Quite often the dog will indicate a generalized pruritus by scratching excessively. In many animals the prepuce becomes flaccid, pendulous and oedematous. There are varying degrees of penile atrophy and loss of libido and energy. Marked deposition of fat of female disposition is often noted. The prostate gland varies from being enlarged and hyperplastic to distinct atrophy. Cyst and abscess formation are not uncommon. Squamous metaplasia of

the prostatic epithelium and of the posterior urethra is a common finding at autopsy.

Mulligan (1944) drew attention to the points of similarity in the symptom-complex of feminization associated with carcinoma of the testis and the effects produced by oestrogens in male dogs. He recorded partial atrophy of the thyroid and adrenal cortex as well as of the non-tumorous testis with arrest of spermatogenesis. Previously, Gardner & De Vita (1940) had shown the inhibition of hair growth in dogs after the administration of oestrogens, whilst other tissue changes and a comprehensive coverage of the literature has been provided by Jabara (1962). The conclusions drawn from these observations were that Sertoli cell tumours of the canine testis may elaborate and secrete oestrogens, thus laying the main emphasis on the Sertoli cell as the site of oestrogen production in the normal testis. Values of up to 70 μg. of a substance, expressed as α-oestradiol, have been extracted from 1 Kg. of Sertoli cell tumour tissue (Huggins & Moulder, 1945). These levels were compared with the 34 μg./Kg. of α-oestradiol which was extracted from canine ovarian tissue at the time of oestrus.

The findings of Mulligan (1944) and Brodey & Martin (1958) of thyroid gland atrophy provoked the latter authors to suggest that the sluggish behaviour observed in these animals may have been caused by the effect of excess oestrogens on this gland. The size of the tumours found in relation to the development of feminization, was commented upon by these authors, being 2 - 3 times that of the tumours in non-feminized dogs. The incidence of hormonal dyscrasia was also found to be higher when the neoplastic testis was ectopic (Scully & Coffin, 1952; Dow, 1962).

Interstitial cell tumours of the canine testis have also been reported to be associated with feminization. Cotchin (1960) found at least 7% of the group of tumours he describes under this heading as being hormonally active (Table 2). Laufer & Sulman (1956) reported an oestrogenic Leydig cell tumour with multiple metastases, and discussed the problem of bisexual hormone production by gonadal cells. Kahan (1955) reported skin troubles, including alopecia, in a 7 yr. old Brittany Spaniel which developed feminization. The dog was castrated and recovery ensued. It appears from the literature that

this particular type of tumour is associated with endocrine changes in man far more frequently than in the dog; an interesting reversal of the situation when compared with the last tumour type.

The correspondence of tumour types in the testes of dog and man has suggested to many that the former could well be used as a model in the study of malignant growths in the latter. Innes (1942) considered that the serious prognostic outlook usually adopted for human cases of testicular tumour is not applicable to dogs, most of which survived for at least two years after orchidectomy without any sign of ill-health. He suggested that a carcinoma in the dog which is histologically identical with the human variety does not behave in the same malignant way. Dow (1962) found metastases in 9% of the canine seminomas he examined, and in 14% of the Sertoli cell tumours (Table 2). Brodey & Martin (1958) found 11% of the latter type to be malignant. The corresponding figures provided by Cotchin (Table 2) are considerably smaller. Coffin, Munson & Scully (1952) considered a malignant Sertoli cell tumour from a dog to be of sufficient interest

to be reported.

Malignancies occurring in human cases of testicular tumour reported by the Testicular Tumour Panel (Pugh & Cameron, 1964) are shown in Table 1. Interstitial cell tumours in this species are generally considered benign but Dalgaard & Hesselberg (1957) estimated that 10% may pursue a malignant course. The reports of Masson & Sencert (1923), Venning (1942), Nation, Edmonson & Hannack (1944), Gharpure (1950), Tedeschi & Burke (1951) would force the conclusion that this type of neoplasm is more malignant in man than in the dog. The seminoma is also more malignant in man and the Sertoli cell tumour less so.

In the experiments described here, all tissues were investigated by <u>in vitro</u> techniques using tissue homogenates or minces (chopped tissue) suspended in solutions containing isotopically-labelled steroids as precursors. The use of homogenates assumes that any chemical reaction occurring in living cells will also occur in a cell-free preparation provided that the conditions are right. As the aim of the homogenate technique is to isolate the reaction

rather than the enzyme, all the tissue must be used and not an extract. The use of minced or chopped tissue, on the other hand, is an attempt to reduce the tissue to such a size not only to permit adequate diffusion in and out of oxygen, solutes in the suspending medium and steroid substrates, but also to provide a uniform tissue suspension of relatively high concentration. Moreover, the use of intact cells assumes that the various cofactors required by the biosynthetic processes under investigation will be produced in the required amounts by the cells themselves. In the case of homogenates, cofactors must be added. A mince does not supply such an organized preparation as does a slice, but the metabolism is nonetheless considered to be that of surviving tissue and reflects qualitatively if not quantitatively that of the original tissue. A further factor for consideration in the case of an intact cell preparation is the permeability barrier that may be offered by the cell membranes.

It is appreciated that the <u>in vitro</u> system is a "closed-system" and thus will not allow the escape of

end-products of metabolism as would occur into the systemic circulation *in vivo*. There is therefore a risk of accumulation of products and the inhibition of enzyme-systems by them. The "profile" of substances formed may thus be distorted. This is less likely to occur, however, with the tracer amounts of substances used, and where precursors are employed with low specific activities (e.g. ^{14}C-labelled steroids) particular attention is paid to keeping the tissue:steroid ratio as high as possible. The *in vitro* technique, nonetheless, gives, probably, the best indications, of the methods at present available, of tissue enzyme activity and inactivity and thus provides an excellent tool for the comparative study of normal and neoplastic cell metabolism as described here.

The determination of metabolites from the incubations has been achieved, in general, by methods similar to those first described by Berliner & Salhanick (1956). These procedures enable the identification of "invisible" quantities of radio-active labelled steroids by indirect methods. In essence the reaction is stopped by organic solvent (acetone) and freezing, followed by the addition of

a known amount (300 μg.) of those steroids to be investigated. Purification of these steroids, which are now "visible" is achieved by chromatography. Proof of identity and radiochemical purity are established by derivative formation, purification and specific activity determination. When the "cold" carrier steroids are added at the outset of extraction, any losses occurring in the radioactive metabolites are reflected exactly in the carriers, and thus percentage conversions may be calculated. For example, if x μc. of radioactive substrate A are incubated and after the reaction has been stopped y mμmoles of product B are added as "cold" carrier, and the mean of three agreeing (within 10%) specific activities of B and two derivatives is z μc./mμmole, then the total radioactivity associated with product B is yz, and the percentage conversion of A to B is $\frac{yz}{x}.100.$

It is appreciated that the term "conversion" only indicates the amount of radioactivity isolated in a particular compound at that moment in time when the reaction is stopped and that more or less

radioactivity may have been isolated with this product depending upon the time in the sequence of biosynthesis that analysis was made. Only a few of the metabolites isolated are end-products in themselves but rather intermediates in biosynthetic chains, but their formation or non-formation, accumulation or non-accumulation provides useful information into the enzyme systems prevailing in a tissue.

In the procedure outlined above one must guess which "carrier" steroids to add and if the precursor were to be converted to a completely unexpected product it could be missed. This product might be picked up by running the total lipid extract of the incubation products on a paper or thin-layer chromatogram and scanning. Again, one must guess the identity of the unknown product giving the peak of radioactivity and one is, of course, limited by the supply of pure carrier steroids available.

The lay-out of this thesis may appear not to conform with the generally accepted form in that there has not been set-aside a section for Results and one for Discussion but rather was it preferred

to present the findings of each investigation in that same section and to discuss their significance at that point. Each investigation thus receives its own debate, in general, while reference to foregoing results ensures continuity.

IV. Experimental.

(A) Source of tissues.

The human tumour was obtained from Mr. J.S.S. Stewart, Surgeon at the Radcliffe Infirmary, Oxford.

The dogs with testicular tumours were referred to me by Miss D.G. Campbell, M.R.C.V.S. and by the Surgery Department, Glasgow Veterinary School.

Normal dog testicular tissue was obtained from an experimental animal and from a dog being destroyed after a road accident.

All tissue was chilled to about 0^{o} after removal from the subject and transferred at this temperature to the laboratory. The time interval from extirpation to incubation was approximately 20 - 30 min. for the dog tissues and 8 hr. for the human.

(B) Preparation of tissues.

(i) Method (1). A mince. The tissue was chopped using the McIlwain & Buddle (1953) apparatus and 1 g. suspended in 13 ml. Krebs-Ringer bicarbonate-glucose medium (see appendix 1.).

(ii) Method (2). An homogenate. The tissue (1 g.) was homogenized at about 0^{o} in 8.0 ml. 0.25M. sucrose containing 0.12M. nicotinamide in a Potter-Elvehjem homogenizer. The homogenate was added to 4.8 ml. of a solution containing the following: 362 μmoles 2-amino-2-hydroxymethyl-1,3-propanediol (TRIS buffer pH 7.4), 360 μmoles KCl, 26.5 μmoles $MgSO_4$, 7.9 μmoles ATP., 72 μmoles potassium fumarate, 1.6 μmoles NADP., 1.6 μmoles NAD., 120 μmoles glucose-6-phosphate and 6.4 Kornberg units glucose--6-phosphate dehydrogenase.

(iii) Method (3). The tissue was chopped at about 0^{o} by hand using a safety razor blade. A known quantity (2 g.) was then suspended in a measured volume (24 ml.) of Krebs-Ringer bicarbonate-glucose solution.

(C) Methods of incubation.

Radioactive steroids (see appendix 2), stored at -15^{o} in benzene:methanol (9:1 v/v), were added to the incubation vessels along with 100 μl. propylene

glycol. The benzene and methanol were evaporated in a stream of air at about 40° leaving the steroids dissolved in a film of propylene glycol. The tissue preparations in their incubation media were added to the flask and, after gassing with 95% O_2, 5% CO_2 in the case of the chopped tissues, placed in a water bath at the requisite physiological temperature and incubated, with shaking for a known length of time from 0.5 to 2.5 hr.

At the end of the incubation period the reactions were stopped by the addition of either 10 ml. benzene: :chloroform (6:1 v/v) or acetone and refrigerating at -15°. The unlabelled carrier steroids were usually added at this stage in a small volume of ethanol.

(D) Methods of extraction of steroids.

(i) Extraction procedure I. The incubation mixtures were homogenised in a Silverson Mixer (Silverson Machines Ltd., London) with 3 x 5 vol. benzene: chloroform (6:1 v/v) followed by 3 x 5 vol. chloroform and finally by 2 x 1.5 vol. ethyl acetate. The combined

extracts were evaporated to dryness under reduced pressure and the residue partitioned between equal volumes of light petroleum (40 - 60°) and 90% methanol in water. The dry residue from the aqueous methanolic extract was taken up in 1 ml. toluene and shaken with 15 ml. N.NaOH. It is appreciated that exposure to this concentration of alkali will decompose some steroids, notably 19-hydroxy C_{19} compounds and oestrogens with Ketol structures, but these were not investigated in the present experiments. The alkali was then extracted with 6 x 15 ml. benzene (Griffiths, Grant & Whyte, 1963). The aqueous phase was adjusted to pH 8 with concentrated HCl and extracted 4 x with equal volumes of ether. The pooled ether extracts were used for the chromatographic investigation of phenolic steroids. The benzene fraction contained the neutral steroids. The aqueous residue from the benzene:chloroform:ethyl acetate extraction was saturated with NaCl and the conjugated steroids extracted with an equal volume of ethyl acetate. This conjugated steroid extract was filtered and evaporated to dryness under reduced pressure.

(ii) Extraction procedure II. The incubation mixtures were homogenized in a Silverson Mixer with 3 x 100 ml. acetone and again with 100 ml. ether: :ethanol (3:1 v/v). After filtering through a scinter glass funnel, the acetone:ether:ethanol mixture was taken to dryness under reduced pressure and the residue partitioned between 70% aqueous methanol and light petroleum (60 - 80°). The alcoholic fraction was reduced to the water phase and the free steroids extracted with 3 x 30 ml. ether. The conjugated steroids were extracted by saturating the aqueous residue with ammonium sulphate and shaking with 3 x 30 ml. ether:ethanol (3:1 v/v) (Edwards, Kellie & Wade, 1953) and then filtering through No. 12 filter paper. The dried ether extract was partitioned between 20 ml. N.NaOH and 2 x 20 ml. toluene. The toluene extracts were pooled and examined for neutral steroids. The NaOH fraction was brought to pH 8 and the phenolic steroids extracted with 3 x 30 ml. ether.

This procedure gives a recovery of 90 - 100% for neutral steroids, 70 - 80% neutral steroid sulphates and 50 - 60% oestrogens (see appendices 3

and 4).

(E) Preparation of chromatograms.

Thin-layers of Merck silica gel $HF_{254/366}$, silica gel G and an anion exchange cellulose (MN--300G/ECTEOLA of Macherey, Nagel & Co., Düren, Germany) were prepared using the Desaga applicator. Suspensions of silicagel (25 g. in 65 ml. water) and of ecteola cellulose (ion exchange capacity 0.35 m. equiv./g.; 12 g. in 60 ml. water) were used to spread, in each case, 5 (20 x 20 cm.) plates. A small amount (100 mg.) of an inorganic phosphor (H.913, Levy West Laboratories Ltd., Harlow) was added to the silica gel G slurries before spreading.

The thin-layer plates were allowed to stand at room temperature for 2 hr. in the case of ecteola and 30 min. for the silica gel before activating in an oven at 50° and 110° respectively. The ecteola--coated plates were found to function equally well if they were merely allowed to stand overnight at room temperature.

Steroids were applied to the plates in ethanol

and the chromatograms developed in the solvent systems shown in Table 4.

(F) Detection of steroids on chromatograms.

The Δ^4-3-oxosteroids were visualised in light of wavelength 254 mμ (Universal U.V. Lamp, Camag, Muttenz, Switzerland) as dark spots on a green background on both types of silica gel.

The Δ^5-3β-hydroxy- and phenolic steroids were located on silica gel $HF_{254/366}$ coated plates in light at 350 mμ, and on silica gel G by spraying standards run at the edges of the plate with a 15% ethanolic solution of phosphomolybdic acid and warming.

The Δ^5-3β-sulphoxysteroids show as fluorescent white spots on a bluish background silica gel $HF_{254/366}$ when exposed to light of wavelength 350 mμ. Standards run at the same time on the silica gel G plates may be located by spraying with the stain described by Eberlein (1965). These conjugates were localized on the Ecteola-coated plates by spraying standard sulphates run alongside with the methylene blue stain of Crepy & Judas (1960).

Testosterone sulphate absorbs light at wavelength 254 mμ when run on either of the silica gel preparations or on Ecteola (without the addition of phosphor).

(G) Elution of steroids from chromatograms.

All steroids were eluted by scraping the chromatographic material containing the steroid on to black glazed paper and transferring to a test tube. Ethyl acetate (4 ml.) or ether was added and the contents thoroughly mixed using a Whirlimixer (Scientific Industries, International Inc. (U.K.) Ltd., England). Water (2 ml.) in the case of free steroids or saturated NaCl solution (steroid sulphates) was added and the tube shaken for several seconds before centrifuging. The upper layer was removed and the aqueous fraction subjected to a second extraction. The extracts were pooled and dried in a stream of air at a temperature not exceeding 50^{o}. The steroid sulphates from the incubation with the interstitial cell tumour (see Section IV, L i) were eluted with methanol which necessitated the use of a correction factor after quantitation with methylene blue (see

appendix 5).

Recovery of steroids by this method is generally of the order of 90 - 100% (see appendices 6 and 7).

(H) Preparation of derivatives.

(i) Oxidation. A chromic acid oxidizing reagent was prepared by the method of Kiliani & Merk (1901) as modified by Griffiths et al. (1963). Concentrated H_2SO_4 (28 ml.) was added to 92 ml. water and the mixture stirred into a solution of 32.2 g. $Na_2Cr_2O_7.2H_2O$ in 70 ml. water and washed in with a further 10 ml. water. The reagent (100 µl.) was stirred into 10 ml. acetone and, after allowing the precipitate to settle, 500 µl. of the resulting mixture were added to the dry steroid and the reaction allowed to proceed for 20 minutes at room temperature. The reaction was stopped by the addition of 2 ml. water and the steroids extracted with ethyl acetate or ether.

(ii) Acetylation. Steroids were acetylated by the procedure of Zaffaroni & Burton (1951). Re-distilled acetic anhydride and pyridine (4 drops of each) were added to the dried steroid and the reaction allowed to

proceed overnight at room temperature in a tightly stoppered test tube. The reaction mixture was then diluted with methanol (1 ml.) and the solvents evaporated completely to dryness in a stream of air.

(iii) Saponification. Steroid acetates were saponified by using a modification (Ward & Grant, 1963) of the method described by Neher, Desaulles, Vischer, Wieland & Wettstein (1958). A solution (250 µl.) of a 2% aqueous solution of K_2CO_3 (w/v) was added to the steroid dissolved in 1 ml. methanol and the mixture allowed to stand overnight at room temperature. Water (2 ml.) was then added and the steroids extracted with ethyl acetate. This procedure gives a 70 - 90% recovery of the free steroid (see appendix 8).

(iv) Reduction. The reduction mixture was made up by dissolving 5 mg. $NaBH_4$ in 10 ml. methanol at 0°. An aliquot (200 µl.) of this solution was added to the dried steroid residue in a tube standing in crushed ice and the mixture maintained at 0° for 45 minutes. This is a modification of the method of

Southcott, Bandy, Newson & Darrach (1956). The reaction was stopped by the addition of one drop of glacial acetic acid and the steroids extracted with ethyl acetate after the addition of water (2 ml.) or saturated NaCl solution in the case of steroid sulphates.

(v) Solvolysis. Steroid sulphates were hydrolysed by the method of Burstein & Lieberman (1958a). The steroids were taken up in 10 ml. water and the pH reduced to 1.0 by the addition of $4N.H_2SO_4$. The solution was saturated with NaCl and the steroids extracted with 2 x 10 ml. ethyl acetate. The pooled extracts were incubated at 50° for 4 hr. and then washed with 5 ml. 5% $NaHCO_3$ and 2 x 2 ml. water. This procedure cleaves the sulphates of phenolic (Sneddon & Marrian, 1963) and neutral steroids but is without effect on glucosiduronates (Jacobsohn & Lieberman, 1962) or phosphates (Burstein & Lieberman, 1958b).

(vi) Methylation. The oestrogens were methylated by the method of Brown (1955). The steroids were

taken up in 50 ml. 1.6% NaOH with the addition of 0.9 g. boric acid to act as a buffer. Dimethyl sulphate (1 ml.) was added by safety pipette and dissolved by shaking. The mixture was incubated for 30 min. at 37° before addition of a further 1 ml. dimethyl sulphate and 2 ml. 5N.NaOH. Solution was again achieved by shaking. There was a further incubation period of 30 min. at 37° and then the mixture was cooled and allowed to stand overnight at room temperature.

Extraction of the methylated oestrogens was achieved, after the addition of 10 ml. 5N.NaOH to the reaction mixture, by shaking with 25 ml. benzene in the case of oestriol and 25 ml. light petroleum (40 - 60°) for the other oestrogens. The extracts were finally washed with 2 x 5 ml. water.

(I) Quantitation of steroids.

(i) Δ^4-3-oxosteroids. Following purification by chromatography, the Δ^4-3-oxosteroids (including testosterone sulphate) were dissolved in 5 ml. ethanol and the optical densities of the

solutions measured at 240 mμ against ethanol in 1 cm. cells of a Unicam SP500 spectrophotometer. Quantitation was achieved by reference to standard solutions. Similar amounts of the chromatographic material were taken through the elution procedure and the optical density readings they gave subtracted from those of the equivalent steroid measured.

(ii) Δ^5-3β-hydroxysteroids. The Δ^5-3β-hydroxysteroids were subjected to treatment with a sulphuric acid-ethanol reagent (Oertel & Eik-Nes, 1959). The reagent was prepared by carefully adding 2 vol. concentrated H_2SO_4 to 1 vol. ethanol with cooling and agitation. The reagent (2 ml.) was added to the dried steroid and the tubes shaken vigorously in a Whirlimixer. Small air-bubbles were allowed to escape and the optical densities (O.D.) of the mixtures measured with light at wavelength 380, 408 and 436 mμ in 1 cm. cells of a Unicam SP600 spectrophotometer against a reagent blank. Standard steroid measurements were taken at the same time and after Allen correction (see below) the amount of steroid present estimated by comparison.

Allen corrected optical densities (Allen, 1950) at the above wavelengths.

Allen corrected O.D. at 408 mμ

$$= O.D._{408} - \frac{O.D._{380} + O.D._{436}}{2}$$

Blanks from the chromatographic material were carried through the same procedure.

(iii) Phenolic steroids. Oestrogens were measured by the method of Brown (1955). The Kober (1931) reagents were made up as shown below:-

Oestriol. 20 g. quinol/litre of 76% H_2SO_4 in water (v/v)

Oestrone. 20 g. quinol/litre of 66% H_2SO_4 in water (v/v)

Oestradiol-17β and oestradiol-17α. 20 g. quinol/litre of 60% H_2SO_4 in water (v/v).

The appropriate quinol-H_2SO_4 reagent (1.5 ml.) for the particular oestrogen was added to the dried steroid in stoppered tubes which were then placed in boiling water for 20 minutes. The tubes were shaken twice during the first 6 minutes of heating. The tubes were then cooled and water (0.5 ml.) added to each oestriol tube, 0.25 ml. to each oestrone tube

and 0.1 ml. to the oestradiol-17β and oestradiol-17α. A Whirlimixer was used to mix the contents of each tube thoroughly after which they were replaced in boiling water for a further 10 minutes. After the tubes had been cooled the optical densities of the mixtures were measured against similarly treated reagent blanks in 1 cm. glass micro-cells of a Unicam SP600 spectrophotometer at the following wavelengths: oestriol and oestrone at 480, 516 and 552 mμ; oestradiol-17β and oestradiol-17α at 480, 518 and 556 mμ.

Optical densities were corrected by applying the Allen correction, e.g. for oestriol.

Allen corrected O.D. at 516 mμ

$$= O.D._{516} - \frac{O.D._{480} + O.D._{552}}{2}$$

The amount of oestrogen present in each tube was found by applying the corrected readings to the particular standard calibration curve prepared with pure oestrogens measured at the same time with the same quinol-H_2SO_4 reagent.

(iv) Δ^5-3β-sulphoxysteroids. The Δ^5-3β-sulphoxysteroids were determined by the methylene blue method of Crepy & Rulleau-Meslin (1960).

The methylene blue reagent was prepared by dissolving 250 mg. of pure methylene blue in 100 ml. water containing 50 gm. sodium sulphate and 10 ml. conc. sulphuric acid. The resulting solution was made up to 1 litre and filtered.

The steroid sulphate was taken up in 0.5 ml. water and shaken vigorously with methylene blue reagent (0.5 ml.) and chloroform (2.0 ml.) and centrifuged. The upper aqueous layer was carefully removed and rejected to allow 1 ml. of the lower organic phase to be pipetted into a tube containing 4 ml. 75% aqueous ethanol. After shaking, the optical densities of the solutions were read using a Unicam SP600 spectrophotometer at the following wavelengths: 625, 650 and 675 mμ against a reagent blank prepared in the same way. The readings were Allen-corrected and the amount of steroid present found by applying the corrected readings to a standard calibration curve drawn up from pure steroid sulphates and read at the same time (see appendix 5).

(J) Measurement of radioactivity in extracts.

Portions of steroid residues to be counted were placed in glass vials of low potassium content (Wheaton Glass Co., Millville, N.J.) and dissolved in 10 ml. toluene containing 3 g./litre of 2,5-diphenyl-oxazole (PPO) and 0.1 g./litre of 1,4-bis-2(4-methyl-5--phenyl-oxazolyl)-benzene (dimethyl POPOP). Radioactivity was determined using a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3214 (Packard Instrument Co., Inc., La Grange, Illinois). Tritium and ^{14}C were determined simultaneously at voltage tap 3.797 with channel I voltage discriminator gate of 50 - 210 and amplifier gate of 25% giving efficiencies of counting of approximately 3.8% for ^{14}C and 18% for ^{3}H. Channel II was set with a voltage discriminator gate of 190 - 1,000 and amplifier gain of 5% giving efficiencies of counting of approximately 33% for ^{14}C and 0% for ^{3}H. Absolute quantities of tritium were calculated using a slight modification (Packard, 1962) of the standard equations of Okita, Kabara, Richardson & LeRoy (1957) and of ^{14}C from direct readings on channel II since, as stated above, no tritium counts appear on this channel

under the conditions specified.

The steroid sulphates were taken up in 1 ml. methanol and 9 ml. scintillator solution (described above) and quenching determined using internal standards (see appendix 9).

Standard equations.

$$R_R = A_1E_{1R} + A_2E_{2R}.$$

$$R_G = A_1E_{1G} + A_2E_{2G}.$$

where R_R = counts/min. in the red channel or channel I.

R_G = counts/min. in the green channel or channel II.

A_1 = activity of isotope I (^3H).

A_2 = activity of isotope II (^{14}C).

E_{1R} = efficiency of counting for isotope I in channel I.

E_{1G} = efficiency of counting for isotope I in channel II.

E_{2R} = efficiency of counting for isotope II in channel I.

E_{2G} = efficiency of counting for isotope II in channel II.

(K) Thin-layer chromatographic separation of steroid sulphates.

During these investigations of steroid biosynthesis

by testicular tumours it has been observed that the tissue preparations possessed the enzyme systems necessary for the sulphation of certain neutral steroids. This observation had not hitherto been reported for testicular tissue and it was considered of sufficient importance to separate these conjugates not only from free steroids and other types of steroid conjugates but also as individual compounds before cleavage of the sulphate radical. Little work has been done on the chromatography of steroid sulphates by thin-layer techniques and what has been reported brought little success in my hands.

An initial isolation of these conjugates from free steroids was achieved using silica gel coated plates in solvent system I (Table 3). At this time of the study, only three steroid sulphates were being investigated, namely the 3β-sulphoxy derivatives of pregnenolone, 17α-hydroxypregnenolone and DHA, which in this system, were found to run virtually together (R_F 0.35) whilst free steroids moved with the solvent front. This system was slightly modified later (solvent system II, Table 3) to one in which the above three conjugates as well as testosterone

Table 3.

Solvent systems used in the separation of steroid sulphates.

A) Using silica-gel-coated plates.

I. tert. butanol/ethyl acetate/5N.NH_4OH (1:1:1 by vol.).

	Rf.
DHA sulphate	0.35
Pregnenolone sulphate	0.35
17α-Hydroxypregnenolone sulphate	0.35

II. tert. butanol/ethyl acetate/5N.NH_4OH (41:50:20 by vol.).

DHA sulphate	0.32
Pregnenolone sulphate	0.32
17α-Hydroxypregnenolone sulphate	0.32
Androst-5-ene-diol-3-sulphate	0.32
Testosterone sulphate	0.32

III. Amyl alcohol/conc. NH_4OH/H_2O (60:30:20 by vol.).

DHA sulphate	0.36
Pregnenolone sulphate	0.12

This is a biphasic system and therefore unreliable.

IV. isopropanol/chloroform/methanol/10 N.NH_4OH.
(50:50:25:10 by vol.).

DHA sulphate	0.5
Pregnenolone sulphate	0.5
17α-Hydroxypregnenolone sulphate	0.5

V. n-butanol/toluene/4N.NH_4OH/methanol
(68:28:40:56 by vol.).

DHA sulphate	0.63
Pregnenolone sulphate	0.63
17α-Hydroxypregnenolone sulphate	0.63

VI. isopropyl ether/tert. butanol/2N.NH_4OH
(18:60:27 by vol.).

DHA sulphate	0.71
Pregnenolone sulphate	0.79
17α-Hydroxypregnenolone sulphate	0.71

B. Using Ecteola-coated plates.

VII. isopropanol/H_2O/Formic acid (65:33:2 by vol.)

DHA sulphate	0.00 - 0.07
Pregnenolone sulphate	0.00 - 0.07
17α-Hydroxypregnenolone sulphate	0.00 - 0.07

All three steroids trailed from the source for about 1 cm.

VIII. Methanol/H_2O/Acetic acid (75:15:10 by vol.).

Results as for system VII.

IX. 1.0 M. acetate buffer pH. 4.75.

DHA sulphate	0.00 - 0.19
Pregnenolone sulphate	0.00
17α-Hydroxypregnenolone sulphate	0.00

X. 0.2 M. $NaHCO_3$.

DHA sulphate	0.41
Pregnenolone sulphate	0.06 - 0.21
17α-Hydroxypregnenolone sulphate	0.00 - 0.26

XI. H_2O.

DHA sulphate	0.13
Pregnenolone sulphate	0.03
17α-Hydroxypregnenolone sulphate	0.00 - 0.08

XII. 0.5 N.NH_4OH.

DHA sulphate	0.27
Pregnenolone sulphate	0.00 - 0.12
17α-Hydroxypregnenolone sulphate	0.00 - 0.17

XIII. 1 N.NH_4OH

DHA sulphate	0.24
Pregnenolone sulphate	0.09
17α-Hydroxypregnenolone sulphate	0.00 - 0.16
Oestrone sulphate	0.15

XIV. 1 M. Urea in 1 N.NH_4OH.

DHA sulphate	0.32
Pregnenolone sulphate	0.14
17α-Hydroxypregnenolone sulphate	0.20 + trail.

XV. 2 M. Urea in 2N.NH_4OH.

DHA sulphate	0.47
Pregnenolone sulphate	0.22
17α-Hydroxypregnenolone sulphate	0.32 + trail
Oestrone sulphate	0.30

XVI. 4 M. Urea in 3N.NH_4OH.

Testosterone sulphate	0.68
DHA sulphate	0.60
Pregnenolone sulphate	0.45
Androst-5-ene-diol-3-sulphate	0.45
17α-Hydroxypregnenolone sulphate	0.00 - 0.45

System II with Ecteola.

tert. butanol/ethyl acetate/5 N.NH_4OH
(41:50:20 by vol.).

Pregnenolone sulphate	0.63
DHA sulphate 17α-Hydroxypregnenolone sulphate Testosterone sulphate Androst-5-ene-diol-3-sulphate	0.43 to 0.58.

sulphate and androst-5-ene-diol-3-sulphate (3β-sulphoxy-androst-5-en-17β-ol) have a common R_F (0.32). The system was prepared by adding, with vigorous shaking, just enough tert. butanol to the ethyl acetate and 5N.NH_4OH to permit complete miscibility of the two phases. The free steroids still move with the solvent front and DHA-glucuronide, at least, is resolved (R_F 0.15).

Resolution of the individual steroid sulphates still, however, remained a problem and Table 3 shows some of the results obtained with silica gel coated plates (systems III to VI). System IV was described by Wusteman, Dodgson, Lloyd, Rose & Tudball (1964). The report by Oertel, Tornero & Groot (1964) gave promise of success but attempts to repeat their work proved quite abortive and, in a personal communication, Dr. Oertel indicated similar difficulty. The material used by these authors, ecteola, has been described more fully previously (see Section IV, E) and initially plates were prepared using their instructions. Eventually these became modified to those described in Section IV, E. Three of the systems described by Oertel et al. (1964) are given

in Table 3 and the R_F values obtained (Systems VII to IX).

Ecteola was continued to be used in attempts to separate the sulphates as shown in Table 3 with systems X to XIII. It was eventually found that the addition of urea to NH_4OH increased the polarity of the system considerably and aided in the separation sought after (systems XIV to XVI). System XVI proved to be the best but there still remained the problem of pregnenolone, 17α-hydroxypregnenolone and DHA sulphates running more or less together. The problem was resolved, however, by an initial chromatographic separation on ecteola-coated plates in system II by which pregnenolone sulphate (R_F 0.63) is resolved from the 3β-sulphates of DHA, androst-5--ene-diol, and 17α-hydroxypregnenolone and testosterone sulphate (R_F 0.43 - 0.58). These latter sulphates are then run on ecteola in system XIII which isolates testosterone (R_F 0.68) and DHA (R_F 0.60) sulphates. Separation of androst-5-ene-diol and 17α-hydroxy-pregnenolone sulphates is achieved by subjecting the eluted mixture from the previous chromatoplate to a

mild oxidation reaction (see Section IV, H i) which converts 3β-sulphoxyandrost-5-en-17β-ol to DHA sulphate which is easily separable from 17α-hydroxypregnenolone sulphate on ecteola in system XIII. These techniques have been reported (Pierrepoint, 1967).

At a later date it was found that an improved resolution and more compact spots could be achieved if the ecteola/water slurry was homogenized for 2 - 3 min. at 6,000 r.p.m. in a 200 ml. Ato-Mix Laboratory Blender (M.S.E., Buckingham Gate, London), and then after spotting the sulphate mixture, placing the plate in a polar lipid solvent system prior to running in system J.

L. Tissue incubations.

(i) An interstitial cell tumour. An investigation of the metabolism, in vitro, of 7α-^{3}H pregnenolone and 4-^{14}C DHA by an interstitial cell tumour of the testis from a man with gynaecomastia.

(a) Clinical data. A 23 yr. old man sought medical advice for impotence of two months duration.

Examination revealed bilateral gynaecomastia with turgid, tender breasts and hard, erect nipples. Both testes were abnormal. The left was soft and small and 3 cms. in length; the right was tense, enlarged and 6 cms. in length, with a hard tender nodule, 3 cms. in diameter, at the lower pole. No striae were present and the nuclear sex was chromosome negative. The daily urinary 17-hydroxycorticosteroid and 17-oxosteroid excretions were 10.0 mg. and 8.3 mg. respectively.

The patient had first noticed an enlargement of the breasts at the age of 17 yr. but his libido and coital performance had apparently been unaffected until 2 mth. prior to examination. The breast enlargement had not regressed after puberty and in the previous year further enlargement had taken place.

The right testis was explored through a groin incision and the presence of a firm 3 cms. diameter spherical nodule was confirmed at the lower pole. This nodule was easily shelled out by blunt dissection together with a thin surrounding layer of normal testicular tissue. Following a frozen section

biopsy report the testis was replaced in the scrotum and the wound closed.

The specimen was an encapsulated tumour of about 2.5 cm. diameter. The cut surface revealed a pale brown, slightly lobulated appearance but was otherwise ~~homologous~~ homogeneous in colour and consistency. A small amount of testicular tissue lay outside the capsule. The patient was seen at follow-up clinic 6 weeks after the operation. The breasts had decreased in size and the left testis was larger than before. After a further 6 weeks, the breasts were no longer turgid or tender and were considered, by the patient, to be normal. He was seen 2 yr. after his operation when normal libido was reported to have returned. He had been having intercourse regularly for 18 mth. and his woman friend was pregnant with what he believed to be his child. Estimations of 17-hydroxy-corticosteroids and 17-oxosteroids in a 24 hr. urine sample at this time were 8.8 mg. and 12.5 mg. respectively.

(b) Histological description. The tumour was composed of almost uniform polyhedral cells closely packed in sheets with a faint trabecular arrangement

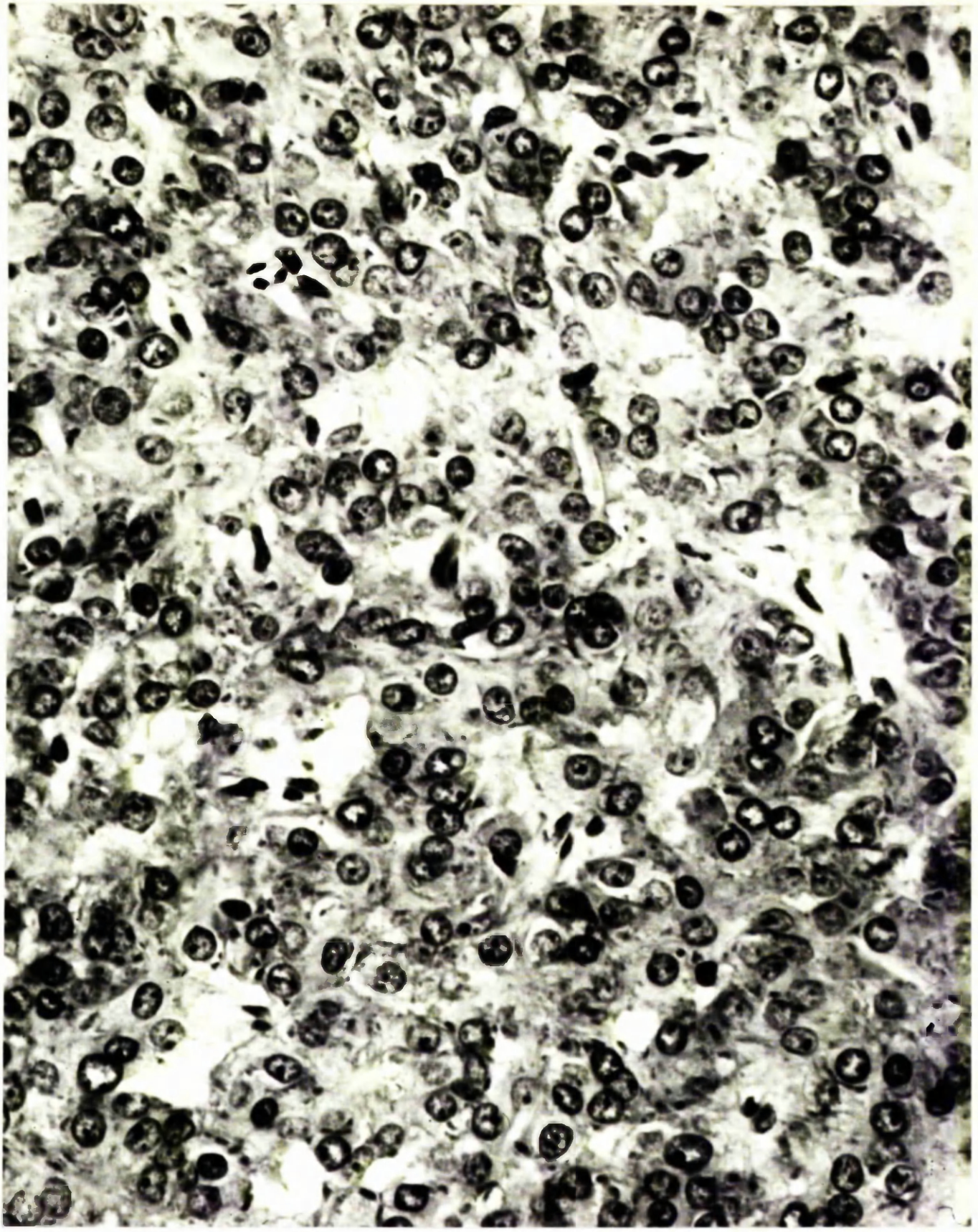


PLATE 1. Human interstitial cell tumour.

(x 500)

(Plate 1). Each cell had one, or occasionally two, large, round or slightly ovoid nuclei with clearly defined nuclear membranes and conspicuous nucleoli. The cytoplasm was eosinophilic and faintly granular, containing numerous finely divided lipoid droplets and brown pigment which did not give the staining reactions of iron or melanin. The tumour was fairly vascular and most of the reticulin was of a perivascular distribution. Mitotic figures were rare and there was a well-defined capsule outside of which was testicular tissue. The tumour was described as an interstitial cell tumour of Leydig cell origin.

In the testicular tissue many of the seminiferous tubules showed a moderate degree of atrophy but in others spermatogenesis was present. There was some thickening of the lamina propria and a moderate amount of interstitial fibrosis. No increase of interstitial cells was seen.

(c) Preparation of tissue and conditions of incubation.

Two types of tissue preparation were employed using Methods (1) and (2) (see Sections IV, B i and IV, B ii, respectively).

In each case, 1 g. of the tumour tissue was incubated at 37^{o} for 2 hr. with 59 mμmoles each of 7α-^{3}H pregnenolone (164 μC/μmole) and 4-^{14}C DHA (36 μC/μmole). The chopped tissue was incubated in O_2:CO_2 (95:5) and the homogenate in air (see Section IV, C).

(d) Extraction and fractionation of steroids.

After incubation, 10 ml. benzene:chloroform (6:1 v/v) and 300 μg. each of oestrone, oestradiol-17β and oestriol, in ethanol, were added. Steroid fractionation was achieved by extraction procedure I (see Section IV, D i).

(e) Investigation of the fractions containing conjugated steroids.

The large number of counts that persisted in the aqueous residue (Table 5) after exhaustive extraction of free steroids from the incubation mixtures with lipid solvents prompted the belief that the tumour tissue had formed water-soluble substances during incubation. The identification of neutral steroid sulphates from adrenal tissue

Table 4. Solvent systems employed in the separation of steroids investigated.

A. Chloroform:acetone (185:15 v/v).

B. Benzene:ethyl acetate (180:20 v/v).

C. Chloroform:ethanol (190:10 v/v).

D. Benzene:ethyl acetate (120:80 v/v).

E. Benzene:methanol (10:1 v/v).

F. Benzene:hexane:ethanol (140:50:10 by vol.).

G. Chloroform:ethanol (10:1 v/v).

H. Cyclohexane:ethyl acetate (1:1 v/v).

I. tert:butanol:ethyl acetate:5N.NH_4OH (1:1:1 by vol.).

J. 4M. Urea in 3N.NH_4OH.

K. Cyclohexane:ethyl acetate (55:45 v/v).

L. Cyclohexane:ethyl acetate:ethanol (45:45:10 by vol.).

M. Cyclohexane:ethyl acetate (140:60 v/v).

N. Cyclohexane:ethyl acetate (150:50 v/v).

O. Ethyl acetate:ether:cyclohexane (40:20:80 by vol.).

P. tert. butanol:ethyl acetate:5N.NH_4OH (41:50:20 by vol.).

Q. Benzene:ethyl acetate (160:40 v/v).

R. Benzene:methanol (170:30 v/v).

S. Chloroform:acetone (170:30 v/v).

T. Hexane:ethyl acetate (1:1 v/v).

Table 5. Recovery of radioactivity in the various steroid fractions

The interstitial cell tumour

Steroid fraction	Radioactivity recovered (dpm.)		% of added radioactivity	
	^{3}H	^{14}C	^{3}H	^{14}C
Homogenate incubation -				
Neutral	11,428,000	1,666,000	53.23	35.57
Phenolic	244,600	86,750	1.14	1.85
Conjugated	3,858,400	2,148,800	17.97	45.87
Mince incubation -				
Neutral	13,305,800	3,284,300	61.98	70.11
Phenolic	271,400	165,000	1.26	3.52
Conjugated	875,600	308,600	4.08	6.59

(Baulieu, 1962; Wallace & Lieberman, 1963; Adams, 1963; Wieland, Levy, Katz & Hirschmann, 1963) and of phenolic steroid sulphates from ovarian (Sneddon & Marrian, 1963) and testicular (Payne & Mason, 1965a) tissues indicated that a similar situation occurring in our experiment might explain the residual radioactivity in the aqueous residue.

A small aliquot of the fractions that would contain conjugated steroids was chromatographed on silica gel G in solvent system I (Table 4) in which the 3β-sulphoxy derivatives of pregnenolone, 17α-hydroxypregnenolone and DHA are not resolved (R_F 0.35) but in which any free steroids remaining move with the solvent front. Standard sulphates and free steroids applied at the edges of the plate were located by spraying over them with a 15% ethanolic solution of phosphomolybdic acid and warming the plate in a hot oven for a few minutes. The areas of silica gel corresponding to the authentic steroids and steroid sulphates were eluted and their radioactive content estimated by liquid scintillation counting (see Section IV, J). The

extraction procedure was shown to be efficient in that the counts at the solvent front, i.e. with free steroids, were negligible whereas those associated with the sulphate area were of sufficient magnitude to warrant further investigation. The remainder of the sulphate band was then subjected to the solvolysis procedure of Burstein & Lieberman (1963a) (see Section IV, H v) and the products formed chromatographed on silica gel in system A (Table 4). The radioactive steroids obtained had the same mobilities as pregnenolone, 17α-hydroxy-pregnenolone and DHA.

At this stage 200 μg. each, of the sulphates of pregnenolone, 17α-hydroxypregnenolone and DHA and unconjugated oestrone, oestradiol-17β and oestriol, were added to the remainder of the two fractions containing conjugated steroids. An aliquot of this fraction from the incubation with minced tissue was chromatographed on ecteola (see in Section IV, E) in system J (Table 4). In this system DHA sulphate is separated from the sulphates of pregnenolone and 17α-hydroxypregnenolone which

are, themselves, not resolved. The DHA sulphate, located by spraying standards at the edges of the plate with methylene blue (Section IV, F) was eluted with methanol and its specific activity determined. Correction was allowed for the effect of ecteola and/or urea on the quantitation of the steroid (see appendix 5).

The remaining portions of both conjugate fractions were then solvolysed as before and the products partitioned between benzene and N.NaOH. Neutral steroids from the benzene phase were chromatographed on thin-layers of silica gel G in system A (Table 4). Pregnenolone, 17α-hydroxy-pregnenolone and DHA, thus separated, were eluted and re-run individually again in system A. After eluting, the acetates of each steroid were prepared (see Section IV, H ii) and chromatographed in system B. Specific activities were obtained for the recovered steroids which were located, as before, with phosphomolybdic acid. Saponification (see Section IV, H iii) yielded the parent steroids which were purified in system A and specific activities

measured. The reduction products of pregnenolone and DHA after chromatography in system A yielded further derivatives for specific activity determinations.

Phenolic steroids from the NaOH phase were examined along with the free steroid phenolic fraction.

(f) Investigation of the fractions containing phenolic steroids.

Oestrone, oestradiol-17β and oestriol in the four phenolic fractions were separated by thin-layer chromatography on silica gel G in system K. The oestriol was further purified, before derivative formation, by chromatography in system L, while the other oestrogens were re-run individually in system K. Elution of these phenolic steroids was achieved by shaking with ether and water (see Section IV, G) and their specific activities assessed. Methyl ethers were prepared (see Section IV, H vi) and purified by thin-layer chromatography, using solvent system B for the derivatives of oestrone and oestradiol-17β and system L for the oestriol

ether. The specific activities of these derivatives were again measured.

(g) Results.

The radioactive content in the various fractions of the products of incubation are shown in Table 5. The conjugated steroid fractions show considerable radioactivity, notably in the case of the incubation with homogenized tissue. Table 6 shows radioactivities associated with the individual steroid sulphates and free steroids investigated. Incubations of both whole cell or homogenized tissue preparations resulted in the formation of radioactive neutral steroid sulphates and in free oestradiol-17β and oestriol. Radioactivity was not found in association with either oestrone or with the solvolysed products of oestrogen sulphates. Evidence for radiochemical purity and identity of steroids investigated is given in Table 7.

(h) Discussion.

In this investigation of oestrogen biosynthesis *in vitro* by a feminizing interstitial cell tumour of

Table 6. Radioactivity associated with steroids isolated from the incubations.

The interstitial cell tumour

Steroid	Total radioactivity associated with the steroid in dpm.		% of added radioactivit	
	^{3}H	^{14}C	^{3}H	^{14}C
Homogenate incubation -				
Pregnenolone sulphate	87,800	-	0.41	-
17αOH-pregnenolone sulphate	60,600	-	0.28	-
DHA sulphate	-	17,300	-	0.37
Oestrone	-	-	-	-
Oestradiol-17β	-	56,000	-	1.20
Oestriol	10,730	-	0.05	-
Mince incubation -				
Pregnenolone sulphate	5,400	-	0.25	-
17αOH-pregnenolone sulphate	2,400	-	0.01	-
DHA sulphate	8,400	3,100	0.04	0.07
Oestrone	-	-	-	-
Oestradiol-17β	-	74,230	-	1.58
Oestriol	13,646	-	0.06	-

Table 7. Evidence for the identification of steroids isolated from the incubated tissue from the interstitial cell tumour

Material investigated and chemical reaction	Chromatographic mobility identical with that of	Solvent system	Specific activity dpm/mμmole			
			Homogenate incubation		Mince incubation	
			^{3}H	^{14}C	^{3}H	^{14}C
Conjugate fraction						
(1) Like pregnenolone sulphate						
-	pregnenolone sulphate		*	*	*	*
Solvolysis	pregnenolone	A	151.8	0	9.1	0
Acetylation	pregnenolone acetate	B	146.5	0	8.7	0
Reduction	pregn-5-ene-3β,20β-diol	A	142.1	0	9.1	0
(2) Like 17α-hydroxypregnenolone sulphate						
-	17αOH-pregnenolone sulphate		*	*	*	*
Solvolysis	17αOH-pregnenolone	A	102.4	0	4.25	0
Acetylation	17αOH-pregnenolone acetate	B	108.0	0	4.10	0
(3) Like dehydroepiandrosterone sulphate						
-	DHA sulphate	J	*	*	13.1	4.8
Solvolysis	DHA	A	‡	27.13	13.3	5.0
Acetylation	DHA acetate	B	67.02	26.15	12.1	4.4
Reduction	androst-5-ene-diol	A	‡	27.51	14.0	5.0
Free steroid - phenolic fraction						
(4) Like oestradiol-17β						
-	Oestradiol-17β	K	0	55.5	0	69.1
Methylation	Oestradiol-17β-3-methyl ether	B	0	46.0	0	65.5
(5) Like oestriol						
-	Oestriol	L	9.9	0	12.9	0
Methylation	Oestriol-3-methyl ether	L	10.6	0	13.2	0

* not measured.

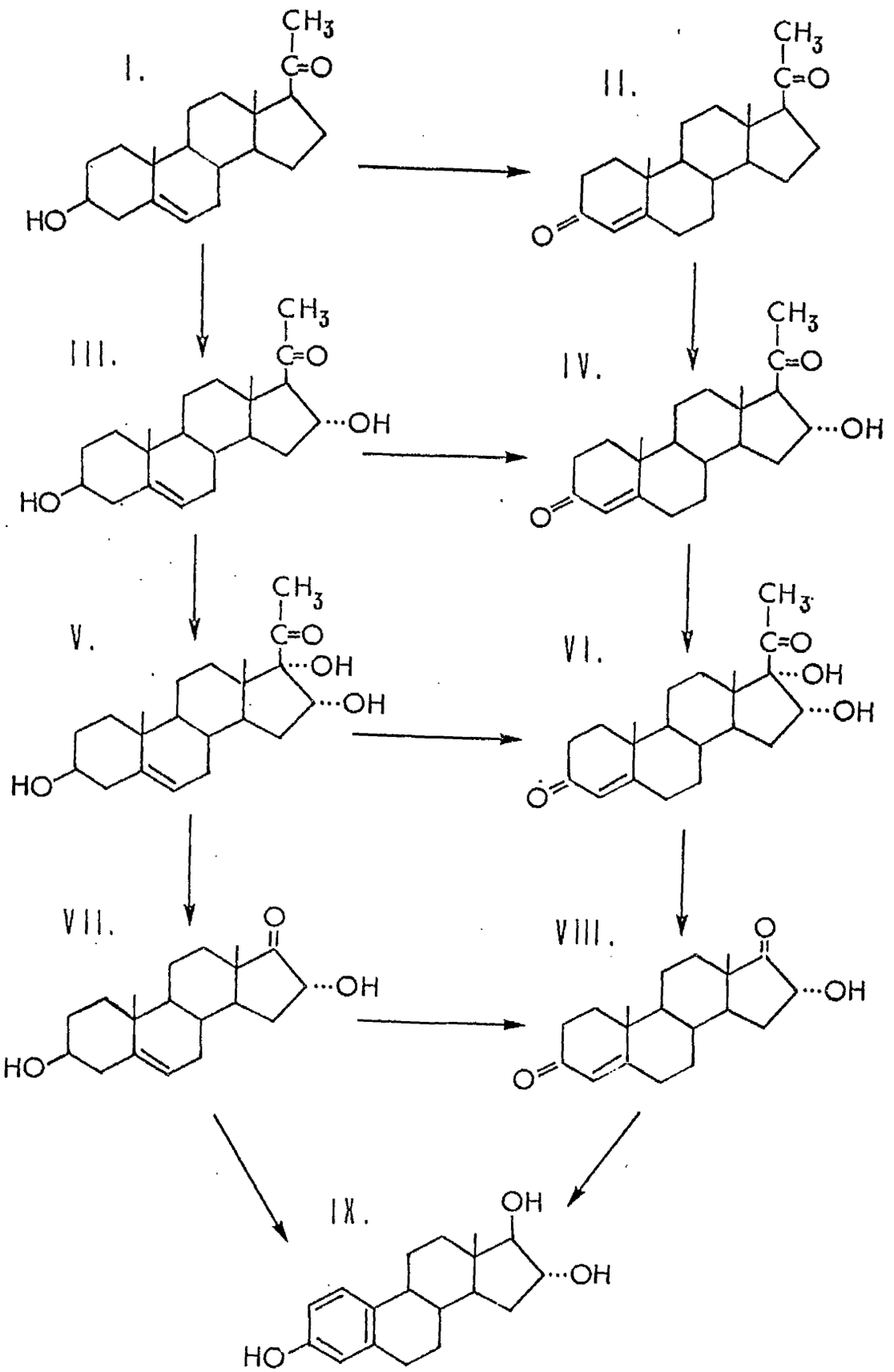
0 no radioactivity.

‡ satisfactory results not obtained.

the testis from a man, oestriol was found to contain tritium derived from 7α-^{3}H pregnenolone but no ^{14}C from 4-^{14}C DHA. Oestradiol-17β, on the other hand, contained ^{14}C but no ^{3}H suggesting that, in this tissue, oestriol was formed from pregnenolone by a pathway not involving DHA or oestradiol-17β. Bolte, Mancuso, Eriksson, Wiqvist & Diczfalusy (1964a) have previously indicated that 16-hydroxylation of oestradiol-17β is not an important pathway in the formation of oestriol by placental tissue *in vitro*, confirming similar observations by Ryan & Engel (1953). A possible precursor of oestriol in the tissue under investigation is 16α-hydroxyprogesterone (see Text fig. 4) which was first shown to be formed *in vitro* by ovarian tissue (Warren & Salhanick, 1961) and which Kadis (1964) has shown to be a precursor of oestriol in the sow ovary. Griffiths *et al*. (1963), Colla, Liberti & Ungar (1966), and Scharma, Dorfman & Southern (1965) have shown the presence of 16α-hydroxylase activity in feminizing abdominal testes. Colla *et al*. (1966) showed a 15% conversion of progesterone to this compound although 16α-

Text–figure 4.

Formation of oestriol via 16 α–hydroxylated neutral steroids.



Key to Text figure 4.

I. Pregnenolone.

II. Progesterone

III. 16α-hydroxypregnenolone.

IV. 16α-hydroxyprogesterone.

V. 3β,16α,17α-trihydroxypregn-5-en-20-one.

VI. 16α,17α-dihydroxypregn-4-ene-3,20-dione.

VII. 16α-hydroxy-DHA.

VIII. 16α-hydroxyandrost-4-ene-3,17-dione.

IX. oestriol.

-hydroxylation has not been shown to be prominent in normal testicular tissue (Viscelli, Hudson & Lombardo, 1965).

No evidence was found for the formation of oestrone or its sulphate by this tissue, nor was it possible to demonstrate the sulpho-conjugation of oestradiol-17β or oestriol although the free steroids in this case were isolated with radioactive labels. Bolte et al. (1964b), however, observed conspicuous differences in the results they obtained with their studies of the foeto-placental unit depending upon which solvolysis technique they employed. They indicated certain limitations in the use of each of the two methods; thus one, involving hydrolysis with a mineral acid gave consistently lower yields of phenolic material and also lower values of oestrone, oestradiol-17β and oestriol-like radioactive material. The other method, involving gel filtration followed by enzymic hydrolysis appeared to yield less neutral material. In the present investigation the mineral acid procedure was used and thus the fact that we found no counts remaining

in the solvolysed "conjugated" phenolic fraction might be attributable, to some extent, to this technique. The absence of ^{3}H from oestradiol-17β that was labelled with ^{14}C indicates that this oestrogen was formed from DHA and not from pregnenolone.

The incorporation of radioactivity into the sulphates of pregnenolone, 17α-hydroxypregnenolone and DHA was observed. The sulphation of steroids by endocrine tissues is now a well-recognized fact. Baulieu (1962) and Wallace & Lieberman (1963) provided evidence of neutral steroid sulphation in the neoplastic adrenal cortex. Adams (1963) and Wieland et al. (1963) reported similar findings with the normal human adrenal cortex. The sulphation of oestrone in vitro by bovine adrenal tissue has been observed (Sneddon & Marrian, 1963). Wallace & Silberman (1964) have demonstrated steroid sulphation in ovarian tissue, and recently Payne & Mason (1965a) showed that testicular tissue could sulphate phenolic steroids. A preliminary account of the work described here has been published

(Pierrepoint, Griffiths, Grant & Stewart, 1965) and in the interim, the sulphation of DHA by normal rat testis (Payne & Mason, 1965b) and of DHA and testosterone by normal human testicular tissue (Dixon, Vincent & Kase, 1965) has been reported.

In the experiment with the whole cell preparation, the presence of ^{3}H as well as ^{14}C in the DHA sulphate indicated that the tissue had the ability to form this conjugated steroid from DHA itself or from pregnenolone. Whether the latter reaction involves the sulphates of pregnenolone and 17α-hydroxypregnenolone (Text fig. 3) as was shown in adrenal tissue (Calvin et al., 1963; Roberts et al., 1964a,b) or whether the biosynthetic sequence proceeds by way of the free steroids is not certain from this investigation. If free ^{3}H DHA is formed, however, it would be surprising if no ^{3}H appeared in the oestradiol-17β. Thus, probably, ^{3}H DHA sulphate is formed by way of the sulphates. Bolte et al. (1964a) found that free DHA is more readily aromatized in the placenta than is DHA sulphate. If a similar situation pertained

to this tumour, it may account for the failure to find ^{3}H in the oestradiol-17β. The formation of neutral steroid sulphates was quite unexpected in this investigation of oestrogen synthesis by an interstitial cell tumour and thus unlabelled carrier steroid sulphates were not added until after the initial extraction procedures. The percentage conversions for the neutral steroid sulphates shown in Table 6 do not, therefore, take into account any losses which may have occurred in those preliminary manoeuvres nor the losses incurred during initial investigation of the conjugate fractions. The percentage conversions indicated are thus minimum values. The conversion figures for the oestrogens, on the other hand, do allow for any such losses and are considered to give a true reflection of the ability of this tissue to form these steroids under the conditions specified.

The experiment with the homogenized tissue showed a higher production of steroid sulphates than did that with the mince. It has been shown by the work of Bernstein & McGilvery (1952a,b), and

De Meio, Wizerkaniuk & Fabiani (1953) that ATP could serve as the source of energy for sulphate activation in the presence of Mg^{++} ions. Both ATP and Mg^{++} ions were added here as co-factors to the incubation with homogenized tissue. Activation is catalysed by the enzyme ATP: sulphate adenylyl-transferase (E.C. 2.7.7.4) which causes the displacement of pyrophosphate from the ATP molecule by the sulphate (Lipmann, 1958) (reaction 1, Text fig. 5). The compound formed, adenosine-5'-phosphosulphate (APS) is entirely without activity as a sulphate donor in enzymatic reactions. The process of sulphate--activation is completed by a second reaction catalysed by the enzyme ATP:adenylylsulphate 3'--phosphotransferase (E.C. 2.7.1.25) whereby the terminal phosphate of a second ATP molecule is transferred to the 3'-position of the APS (Robbins & Lipmann, 1957) (reaction 2, Text fig. 5).

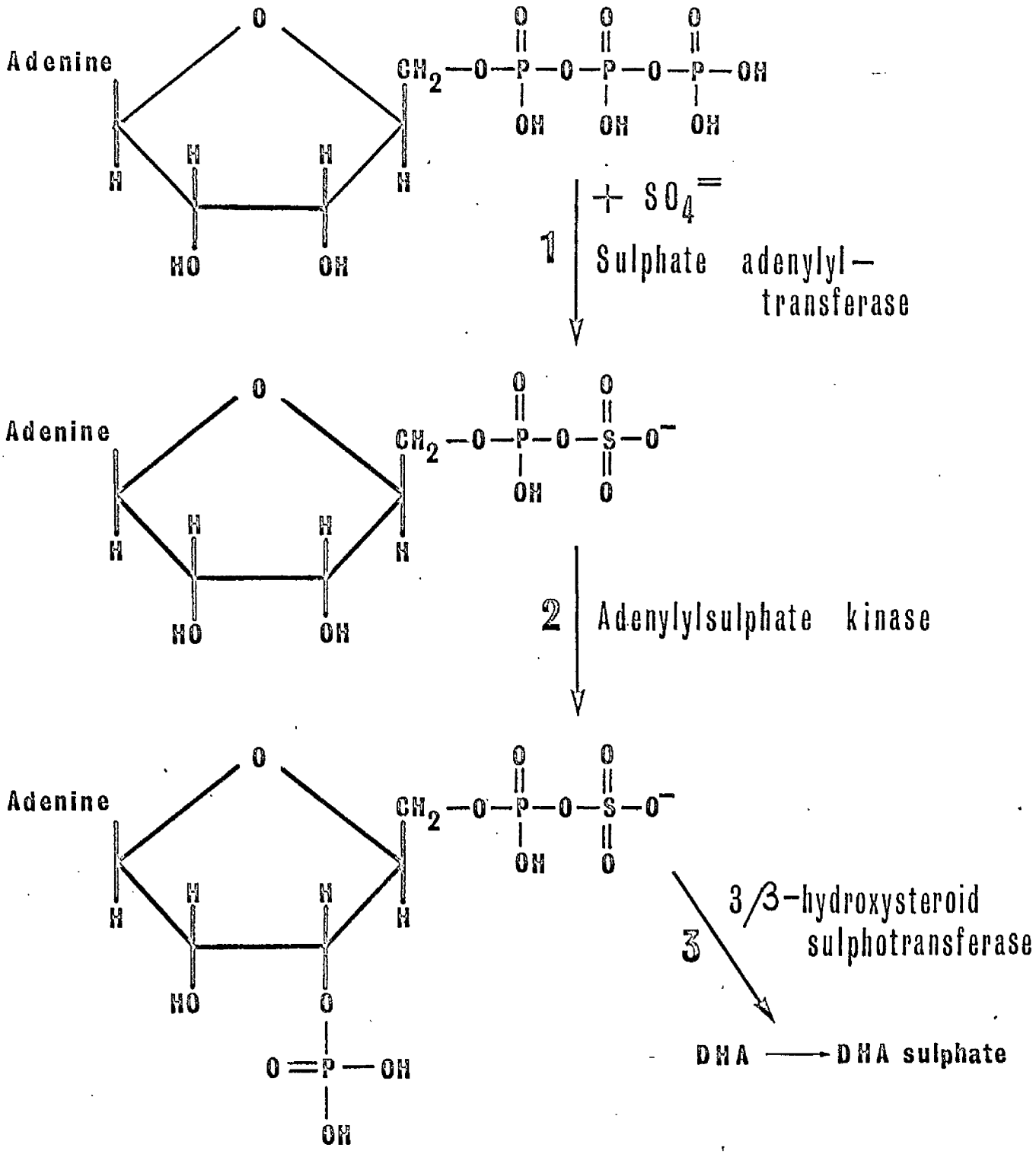
Thus $ATP + S \longrightarrow APS + PP$

$ATP + APS \longrightarrow PAPS + ADP + H^{+}$

The final product, adenosine-3'-phosphate-5'-

Text–figure 5.

Biosynthesis of steroid sulphates.



-phosphosulphate (PAPS) appears to be the general sulphate donor in biological reactions and carries the sulphate in the form of a mixed anhydride.

The transference of sulphate from PAPS to an acceptor (steroid) is catalysed by enzymes called sulphotransferases, which, for a steroid such as DHA would be 3'-phosphoadenylylsulphate:3β-hydroxy-steroid sulphotransferase (E.C. 2.8.2.2.) (reaction 3, Text fig. 5). The activation of the sulphate is catalysed by the same enzymes regardless of the type of sulphate acceptor involved, whereas the sulphate transfer requires more or less group specific or acceptor specific enzymes (Nose & Lipmann, 1958).

The formation of neutral steroid sulphates by this type of tissue is of particular interest in that these conjugates have been incriminated in the production of both virilizing and feminizing hormones. The efficiency of DHA sulphate as a precursor of oestrogens has been demonstrated in vitro with placental tissue (Morato, Lemus & Gual, 1965) and in vivo in pregnant women (Baulieu & Dray, 1963; Siiteri & MacDonald, 1963; Warren

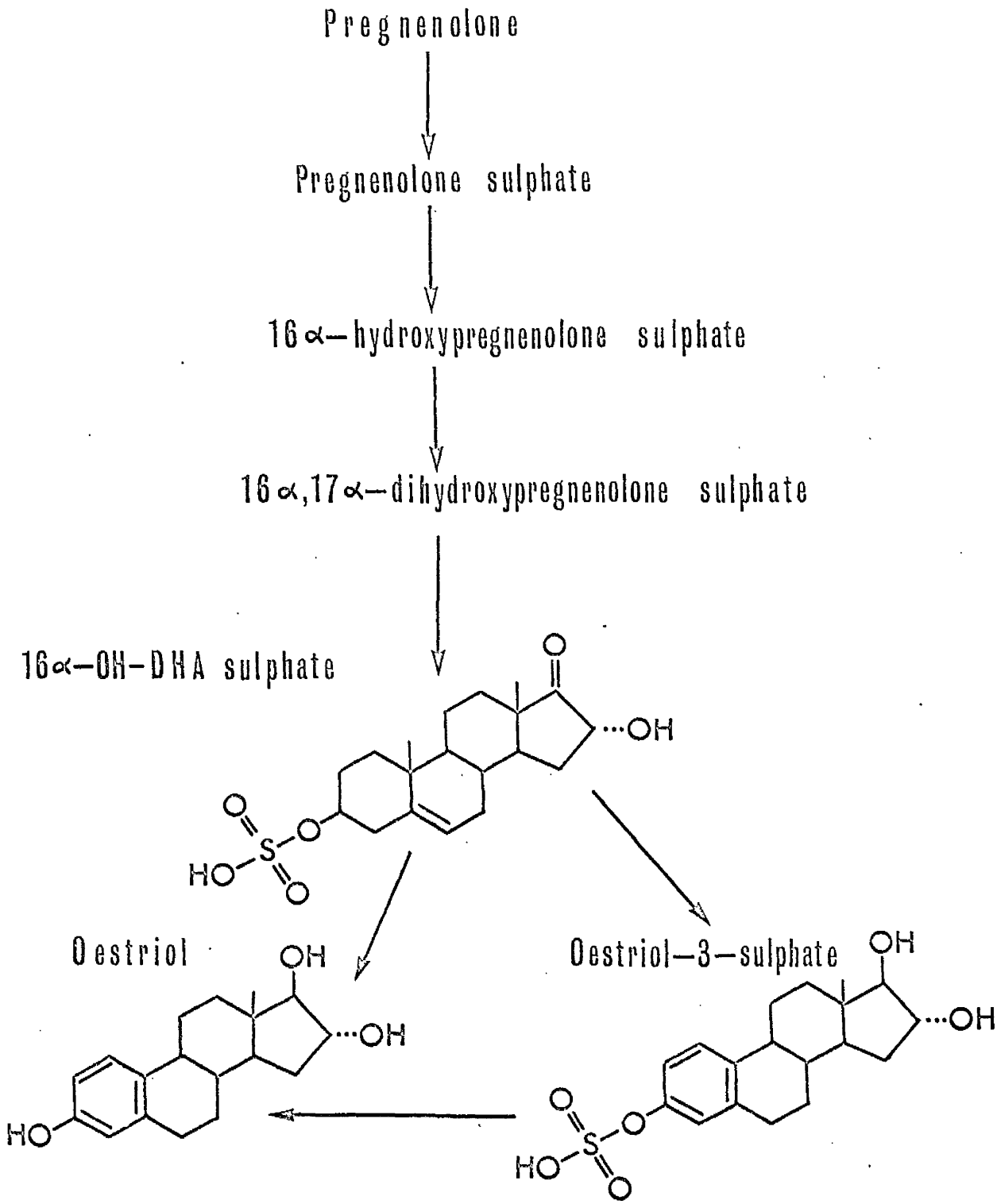
& Timberlake, 1964; Bolte et al., 1964b), and as a precursor of testosterone in the dog testis (Aakvaag et al. 1964). That steroid sulphates are not merely metabolic end-products of steroid metabolism is now accepted and Calvin & Lieberman (1966) have presented evidence of the complex metabolism of pregnenolone sulphate in man. These authors indicate that the metabolic function of the sulphate ester in the over-all process of oestrogen synthesis may be to serve as a biochemical protective group, since DHA, androstenedione and testosterone, which have considerably shorter half-lives (Sandberg & Slaunwhite, 1956; Sandberg, Gurpide & Lieberman, 1964) than has DHA sulphate, are less efficiently converted to oestrogens than is the sulphated steroid when administered to pregnant women (Baulieu & Dray, 1963; Siiteri & MacDonald, 1963). Evidence for the formation of DHA sulphate from cholesterol sulphate via sulphated intermediates (Roberts et al., 1964a,b) has been commented upon earlier in an interpretation of the ^{3}H DHA sulphate formed in the present investigation. It is worthy of note, however, that Calvin & Lieberman

(1966) isolated 3β-sulphoxy, 16α-hydroxyandrost-5--en-17-one (16α-hydroxy DHA sulphate) bearing ^{3}H and ^{35}S in a ratio similar to that of the injected cholesterol-^{3}H sulphate-^{35}S, indicating a direct transformation without loss of the sulphate group. A similar sequence occurring in this tissue via 16α-hydroxylated neutral steroid sulphates might provide an alternative explanation for the tritiated oestriol formation (Text fig. 6). Siiteri & MacDonald (1963) found indications that oestriol may be derived from DHA sulphate by a biochemical pathway which did not involve oestrone or oestradiol-17β but rather via a 16α-hydroxylated intermediate. Corpechot & Baulieu (1964) demonstrated the direct conversion of DHA sulphate--^{35}S-^{3}H <u>in vivo</u> to 3β-sulphoxyandrost-5-ene-16α,17β--diol-^{35}S-^{3}H indicating the significance of the metabolism of a steroid conjugate for the biosynthesis of oestriol.

The presence of sterol sulphatase (E.C. 3.1.6.2.) activity in human testicular tissue has been established (Burstein & Dorfman, 1963). It is therefore of considerable interest to speculate on

Text-figure 6.

Possible pathways to the formation of oestriol via steroid sulphates.



the part that sulphation may play in the feminization of a patient with an interstitial cell tumour of the testis.

A communication on these findings has been published (Pierrepoint, Griffiths, Grant & Stewart, 1966).

(ii) Normal testicular tissue (1).

An investigation of the effect of time on the metabolism, in vitro, of 4-^{14}C DHA and 7α-^{3}H DHA sulphate by normal canine testicular tissue.

(a) Preparation of tissue, incubation procedure and method of extraction of steroids.

A testis was surgically excised from a dog under general anaesthesia (intravenous thiopentone Na/ /fluothane gas and oxygen). The organ, weighing 18.5 g., was prepared for incubation by Method 3 (see Section IV, B iii) and three portions of 2 g. were each suspended in 24 ml. Krebs-Ringer bicarbonate glucose solution and incubated with 72.7 mμmoles each of 4-^{14}C DHA (27.5 μC/μmole) and 7α-^{3}H DHA sulphate (206.3 μC/μmole) at 37° in an atmosphere of O_2:CO_2 (95:5). After half-an-hour's incubation, one flask was removed from the water bath and the reaction stopped by the addition of 10 ml. acetone and cooling to -15°. This was

repeated at 1.5 hr. and 2.5 hr. for the second and third flasks respectively. The following unlabelled carrier steroids, 300 μg. of each, were added to each of the three flasks; DHA sulphate, DHA, androst-5-ene-diol, androstenedione and testosterone. Flask 3 (2.5 hr. incubation time) received in addition 300 μg. of unlabelled testosterone sulphate. The contents of each flask were thoroughly mixed. Steroids were extracted from the incubation mixtures in each case, by Extraction Procedure II (see Section IV, D ii) providing three fractions from each, containing neutral, phenolic and conjugated steroids respectively.

(b) Investigation of the fractions containing neutral steroids.

These fractions were chromatographed initially on plates coated with silica gel $HF_{254/366}$ in solvent system A (Table 4). This type of silica gel was used from hereon-in unless otherwise stated. The steroids eluted from this first run were examined as described below.

The DHA, after elution, was acetylated and chromatographed in system B. Elution, saponification and chromatography of the original steroid again in system A was considered sufficient purification prior to the first specific activity determination. The acetate was again prepared from a part of the product and run in system B, whilst the reduction product, androst-5-ene-diol, prepared from a further aliquot, was chromatographed in system C. Specific activities of these derivatives were determined.

The androst-5-ene-diol was acetylated and the products resolved by chromatography in system E. Androst-5-ene-diol diacetate was located, eluted and saponified to give the original steroid. Purification of this was achieved in system C before measurement of its specific activity. The diacetate was again prepared and run in system E to provide a derivative for a second specific activity.

The androstenedione was purified by subjecting it to an acetylation reaction and re-running in system A. A specific activity was measured on the

eluted unchanged compound. Reduction to testosterone and subsequent acetylation of this product provided two derivatives for specific activity determination after each had been chromatographed in system A.

The testosterone was converted to the acetate and chromatographed in system A. Elution and saponification yielded the original steroid which was purified in the same system. The specific activity was measured. The acetate and oxidized product were prepared and measured after chromatographing each in system A.

(c) Investigation of the fractions containing conjugated steroids.

The procedure for the handling of steroid sulphates has been described (see Section IV, K).

In brief, the three fractions containing conjugated steroids were chromatographed on silica gel $HF_{254/366}$ in solvent system I (Table 4). Three-quarters of the eluted steroid sulphate fraction were subjected to the solvolysis procedure of Burstein & Lieberman (1958a) (see Section IV, H v)

and the resulting free steroids chromatographed in system A after the addition of 200 μg. of non--radioactive androst-4-ene-diol to the fraction from the 2.5 hr. incubation. DHA was eluted from the chromatograms of incubations (1) and (2) and DHA, testosterone and androst-4-ene-diol from that of incubation (3). The androst-4-ene-diol was oxidized to androstenedione and purified in system A before determining its specific activity. The other two steroids were dealt with exactly as described above for the same steroids from the neutral fractions.

The remaining unsolvolysed portion of each fraction was chromatographed on ecteola in system J and DHA and testosterone sulphates isolated and their specific activities determined.

The phenolic fractions were not examined other than assessing the total radioactivity in this fraction from the 2.5 hr. incubation (Table 8).

(d) Results and discussion.

The radioactive content of the various fractions from the three incubations and the radioactivity associated with the individual steroid sulphates and free steroids investigated are shown in Table 8. Evidence for the radiochemical purity and identity of the steroids investigated is given in Table 9. A graphic representation of the formation of testosterone, androst-5-ene-diol and androstenedione from 4-^{14}C DHA and 7α-^{3}H DHA sulphate is provided by Graphs 1 and 2 respectively. The rates of transformation of DHA and DHA sulphate as indicated by loss of radioactivity with time are shown by Graph 3.

It may be seen from Graph 3 that DHA is at first metabolized rapidly; 20% of this steroid having been transformed in the first 30 min., and after 1.5 hr. only half of this substrate remained unchanged. DHA sulphate, on the other hand, is only very slowly metabolized and even after the 2.5 hr. incubation period barely 7% of it had been transformed.

The interconversion of these two compounds is

Table 8. Recovery of radioactivity in the various fractions and the total radioactivity (dpm x 10^{-3}) associated with the steroids isolated from the incubations with normal canine testicular tissue (1). Percentages of the initial radioactivity are shown in parentheses

	From 7α-^{3}H DHA sulphate						From 4-^{14}C DHA					
	After 0.5 hr.		After 1.5 hr.		After 2.5 hr.		After 0.5 hr.		After 1.5 hr.		After 2.5 hr.	
Neutral steroid fraction	65.7	(2.19)	1,345.6	(4.53)	2,546.1	(8.57)	5,036.5	(92.5)	3,724.2	(68.4)	4,478.4	(82.2)
Phenolic steroid fraction	*		*		30.09	(0.10)	*		*		191.59	(3.52)
Conjugated steroid fraction	24,321	(81.9)	18,319	(61.7)	21,875	(73.6)	22.43	(0.41)	35.78	(0.66)	81.05	(1.49)
DHA	350.4	(1.12)	1,307.6	(4.40)	1,659.8	(5.59)	4,351.9	(79.9)	2,756.5	(50.6)	2,213.4	(39.2)
androst-5-ene-diol	15.73	(0.05)	162.0	(0.55)	386.38	(1.30)	369.93	(6.79)	642.42	(11.8)	923.3	(17.0)
androstenedione	4.80	(0.02)	39.23	(0.13)	52.24	(0.18)	109.41	(2.01)	183.6	(3.37)	150.84	(2.77)
testosterone	6.90	(0.02)	140.4	(0.47)	318.8	(1.07)	249.0	(4.57)	867.1	(15.9)	1,185.9	(21.8)
DHA sulphate	30,000	(100)	27,988	(94.2)	27,102	(91.2)	1.09	(0.02)	3.023	(0.06)	5.454	(0.10)
testosterone sulphate	*		*		0		*		*		1.385	(0.03)
androst-4-ene-diol sulphate	*		*		0		*		*		0	

* not investigated.

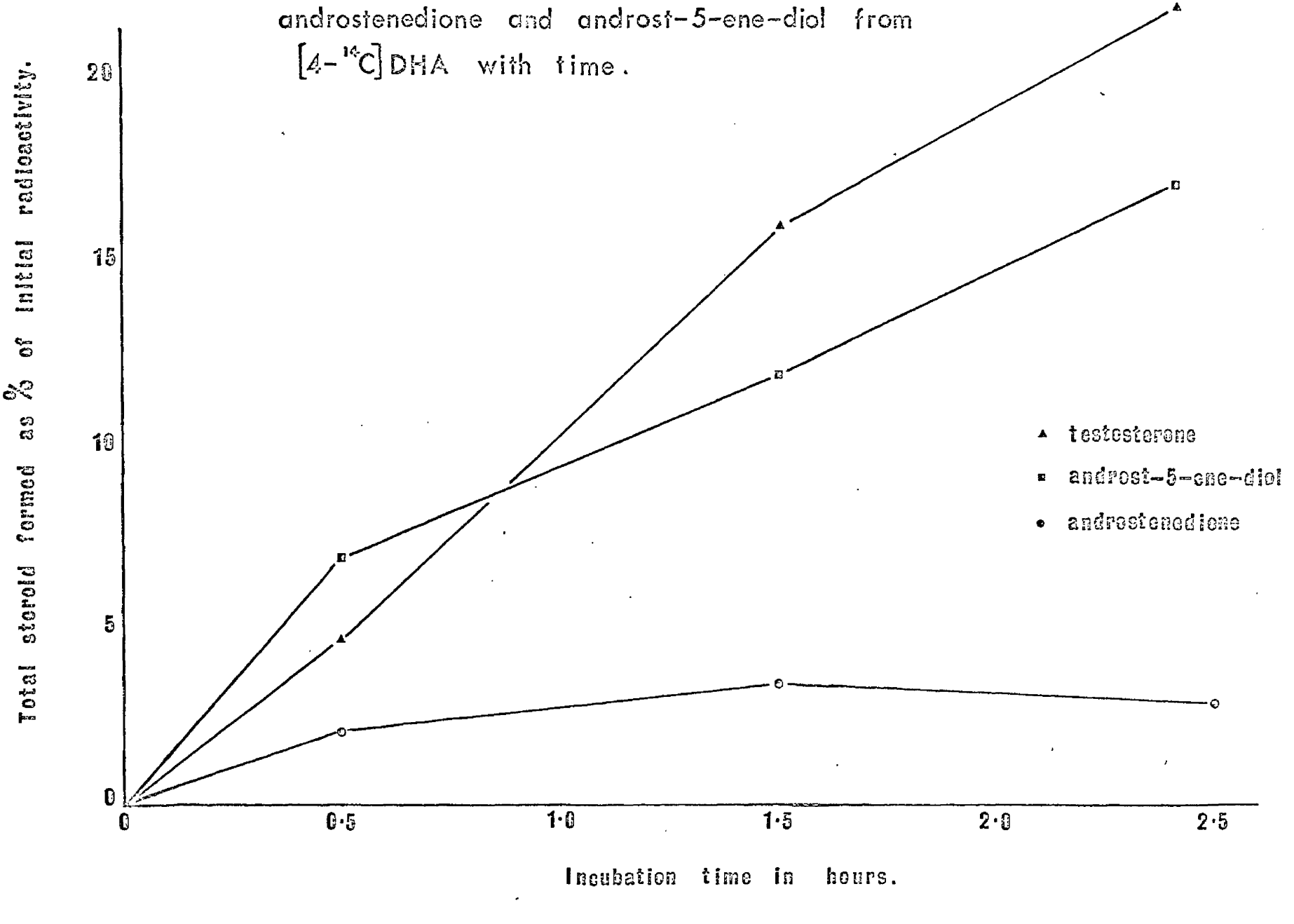
Table 9. Evidence for the identification of steroids isolated from the three incubations with normal testicular tissue (1)

Steroid investigated and the chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	Specific activities (dpm/mμmole) After 0.5 hr. ^{3}H	After 0.5 hr. ^{14}C	After 1.5 hr. ^{3}H	After 1.5 hr. ^{14}C	After 2.5 hr. ^{3}H	After 2.5 hr. ^{14}C
1. DHA								
-	DHA	A	352.0	4,117.1	1,198.4	2,455.6	1,560.4	2,190.9
acetylation	DHA acetate	B	336.0	4,073.2	1,283.6	2,741.3	1,615.3	1,994.6
reduction	androst-5-ene-diol	C	321.2	4,343.2	1,283.8	2,741.7	1,604.0	2,189.3
2. androst-5-ene-diol								
-	androst-5-ene-diol	C	346.5	15.2	161.4	627.1	378.5	887.8
acetylation	androst-5-ene-diol diacetate	E	368.7	15.2	151.8	614.8	368.5	897.1
3. androstenedione								
-	androstenedione	A	4.2	104.0	36.2	178.7	51.6	144.4
reduction	testosterone	A	4.3	104.1	36.6	175.7	50.5	146.3
acetylation	testosterone acetate	A	3.9	104.7	39.4	171.0	47.3	140.6
4. testosterone								
-	testosterone	A	6.4	238.6	137.3	829.3	296.3	1,156.0
acetylation	testosterone acetate	A	7.2	243.7	128.3	852.8	296.3	1,137.0
oxidation	androstenedione	A	6.2	234.9	138.8	815.2	325.4	1,122.0
5. DHA sulphate								
-	DHA sulphate	J	*	*	*	*	33,189	‡
solvolysis	DHA	A	39,450	1.65	37,426	4.61	33,894	6.89
acetylation	DHA acetate	B	38,370	1.10	34,243	3.45	36,491	7.44
reduction	androst-5-ene-diol	C	40,890	1.52	37,484	3.74	37,357	6.95
6. testosterone sulphate								
-	testosterone sulphate	J	*	*	*	*	‡	‡
solvolysis	testosterone	A	*	*	*	*	0	1.78
acetylation	testosterone acetate	A	*	*	*	*	0	1.85
oxidation	androstenedione	A	*	*	*	*	0	1.76
7. androst-4-ene-diol sulphate								
solvolysis and oxidation	androstenedione	A	*	*	*	*	0	0

* not measured.

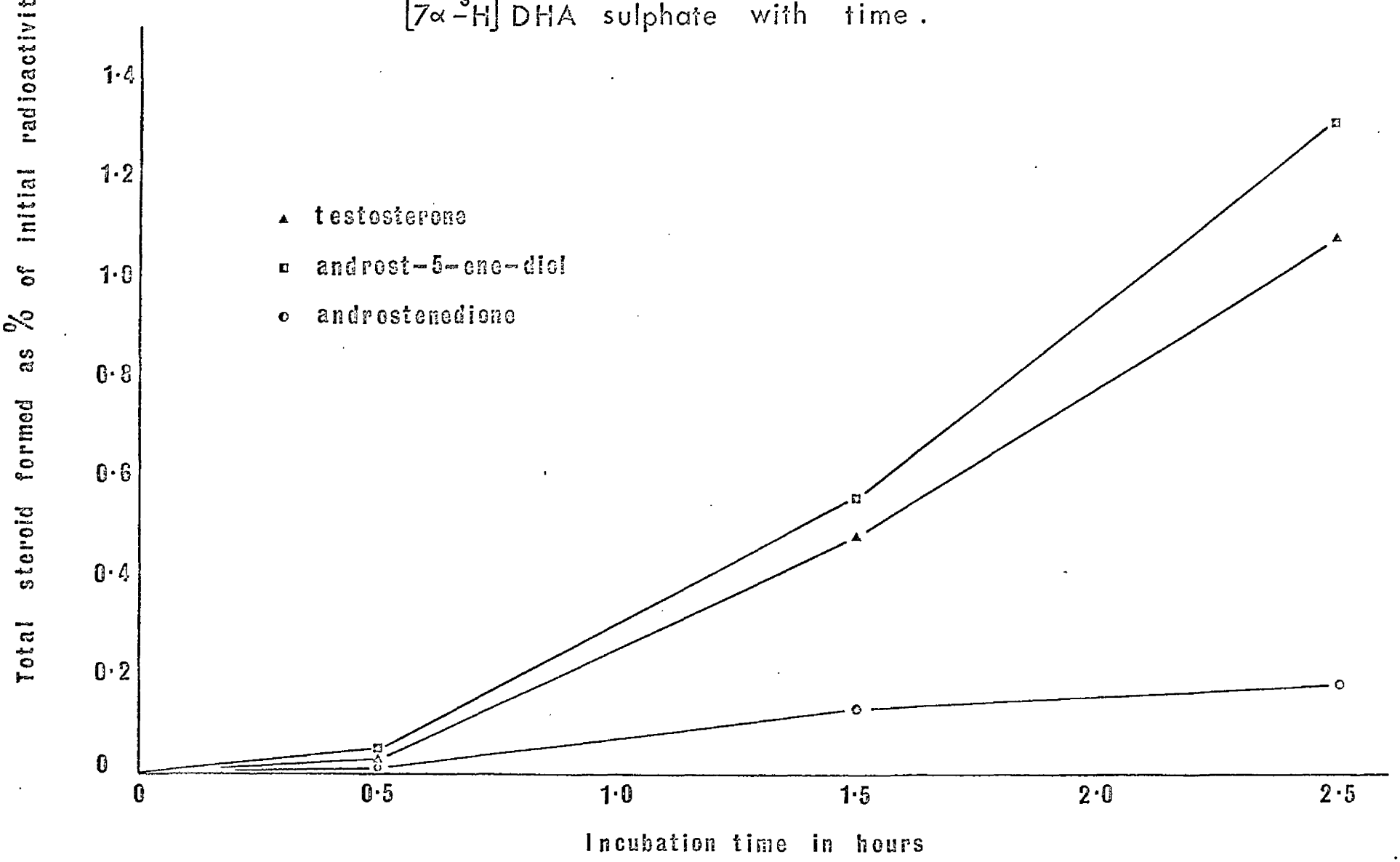
‡ unsatisfactory result.

Graph 1. The graphic representation of the formation of testosterone, androstenedione and androst-5-ene-diol from [4-^{14}C] DHA with time.



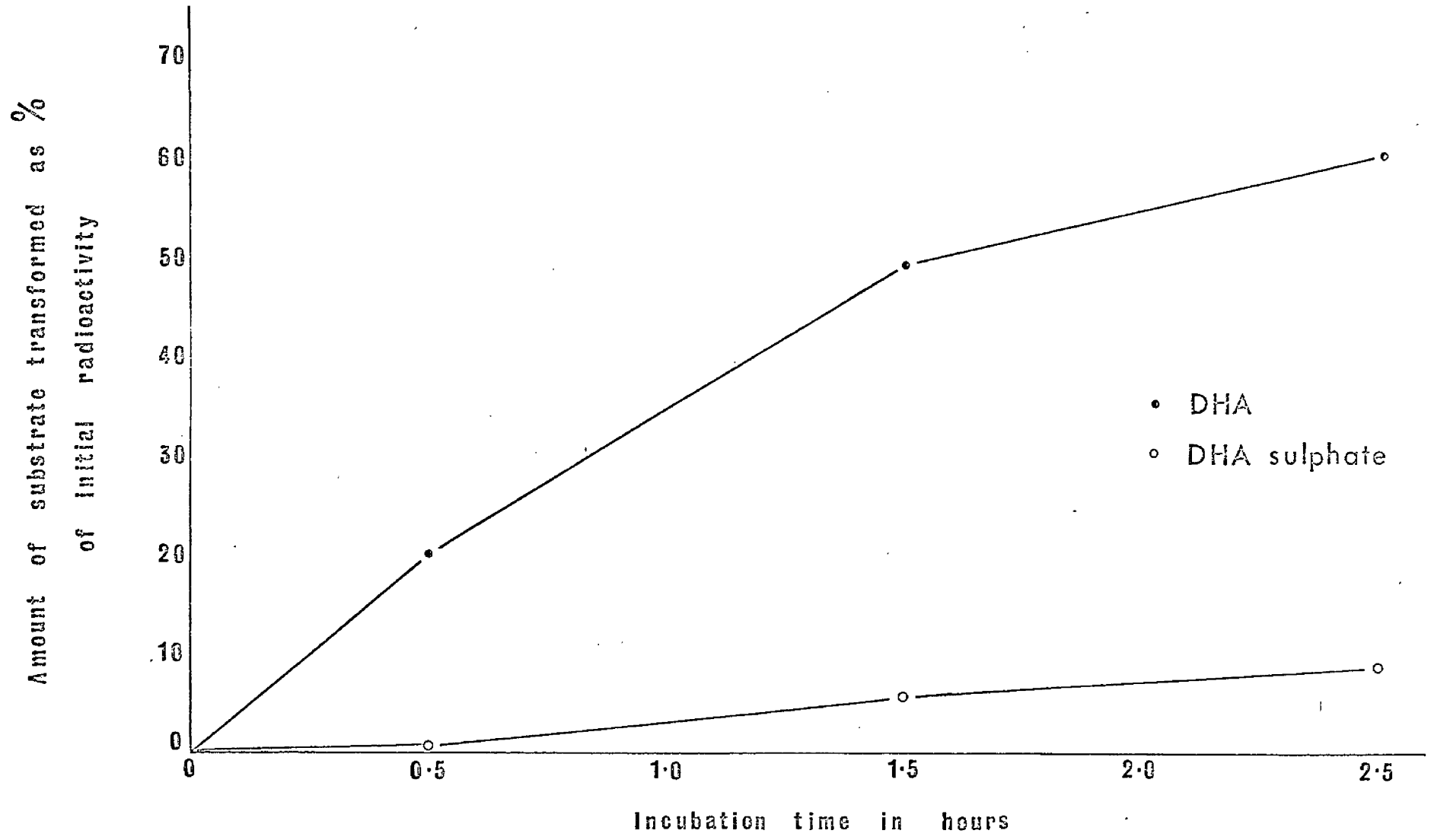
Graph 2.

The graphic representation of the formation of testosterone, androstenedione and androst-5-ene-diol from [7α-^{3}H] DHA sulphate with time.



Graph 3.

The graphic representation of the rate of transformation of [4-^{14}C] DHA and [7α-^{3}H]DHA sulphate.



of interest. It may be seen from Table 8 that the incorporation of ^{3}H into DHA exceeds by at least 50 times, at any stage of the experiment, the reciprocal incorporation of ^{14}C into DHA sulphate. This latter incorporation was, at 2.5 hr., only 0.1% of the original ^{14}C. Despite the low rate of transformation of the DHA sulphate, the experiment shows for the first time the ability of canine testicular tissue to use this conjugate in the synthesis of testicular androgens, in vitro. Aakvaag et al. (1964) demonstrated the conversion of DHA sulphate to testosterone and androstenedione by the dog testis in vivo after pretreating the animals with human chorionic gonadotropin, and declared a minimum conversion figure of 2%. The results presented here confirm this work and in addition show the production of androst-5-ene-diol from DHA sulphate in slightly greater quantities than either testosterone or androstenedione. The total production of the three ~~being~~ is of the order of 2 to 3% of the sulphate presented to the tissue.

The conversion of 4-^{14}C DHA to 4-^{14}C testosterone sulphate is noted. The formation of this

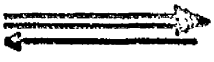
conjugate was low (0.03%) and it is assumed that it was formed by the sulphation of testosterone and that the ^{3}H content of this steroid was too low to be assessed by the techniques adopted here. Dixon et al. (1965) have shown that homogenates of normal human testes will convert DHA and testosterone to their sulphate esters and concluded that testosterone sulphate was formed directly from free testosterone. The search for an androst-4-ene-diol sulphate proved abortive and no radioactivity was isolated after addition of the free steroid to the conjugate fraction, solvolysis and subsequent oxidation to androstenedione.

The biotransformation of DHA favours, initially, the synthesis of androst-5-ene-diol but after about 50 min. incubation period, it may be seen from Graph 1 that the formation of testosterone exceeds that of androst-5-ene-diol. Androstenedione, with respect to either precursor, at no time features as a major metabolite. It has a steady rate of formation from DHA up to 1 hr. at which stage the quantity of this steroid present reaches a plateau (3.4%) due either to non-formation, or its production

and further metabolism proceed at equal rates. A similar picture is seen in its formation from DHA sulphate although the rate of synthesis is much less and the level is still increasing after 2.5 hr. (0.18%). It may be argued that the amount of this steroid does not reach high levels as it is being converted to testosterone as quickly as it is formed, and the present experiment casts little light on this problem, nor was it designed to do so. It is, however, of interest that the production of testosterone should parallel almost exactly that of androst-5-ene-diol from both precursors. With regard to their respective formation from 4-^{14}C DHA it can be seen from Graph 1 that androst-5-ene-diol, being formed first by reduction of the 17-oxo group of DHA, is present in excess of testosterone until almost 1 hr. of incubation, at which time one could assume that its own further metabolism by the enzyme--complex 3β-hydroxysteroid dehydrogenase-isomerase to testosterone just exceeds its own formation, after which point-in-time the rates of production and metabolism of androst-5-ene-diol remain constant.

At the 2.5 hr. period almost 22% of the DHA has been converted to testosterone and 17% to androst--5-ene-diol with less than 3% remaining as androstenedione. Savard & Dorfman (1954) reported in their abstract that androst-5-ene-diol was converted to testosterone in vivo by the rat testis.

Slaunwhite & Burgett (1965) found that rat testicular tissue converted DHA to androstenedione very quickly and efficiently but that the transformation to androst-5-ene-diol was trivial. They suggest that while DHA is oxidised to androstenedione, DHA sulphate may be reduced to androst-5-ene-diol-3-sulphate which, on hydrolysis, and oxidation, forms testosterone. This sequence of reactions may well hold also for the dog testis but, as the results show, it is of little importance, providing, at best, 0.13% conversion to testosterone. These same authors found that an equilibrium between androstenedione and testosterone was not reached even after 3 hr. incubating with testicular tissue and that the oxidized form predominated.

Thus Testosterone  Androstenedione.

This is shown not to be the case with dog testis, and even though equilibrium had not been reached the testosterone production rate was greatly in excess of that for androstenedione.

Gower (1966), also working with rat testicular tissue in vitro, found that after 1 hr. virtually all the incubated 4-^{14}C DHA had been transformed. In a 4 hr. incubation he found only a 0.05% conversion to androst-5-ene-diol and concluded that little or none of this compound was formed although in his discussion he suggests that it is efficiently converted to testosterone. He also suggests that the high activity he found in androstenedione relative to that in the androst-5-ene-diol isolated after a 4 hr. incubation period confirms that the conversion of DHA to the former is very rapid whereas the conversion of DHA to androst-5-ene-diol is trivial.

The comparison of these results serves to indicate differences in steroid metabolism by the testes of two species. The results achieved in the present investigation indicate the comparative

ability of dog testicular tissue to metabolize DHA and its sulphate and provide some information on the rate of production of some testicular androgens. These results will be used later to emphasize the abnormal steroid biosynthesis encountered in two testicular tumours from dogs showing clinical endocrine dysfunction (see Sections L, v and L, vi).

(iii) Sertoli cell tumour (1).

An investigation of the metabolism, in vitro, of 7α-^{3}H pregnenolone, 4-^{14}C DHA and 4-^{14}C testosterone by a Sertoli cell tumour of the testis from a dog showing signs of feminization and alopecia.

(a) Clinical data.

The patient, an 11 yr. old West Highland White terrier, was submitted for veterinary advice because of widespread bilateral alopecia. Enlargement of the nipples and a large swelling in the scrotum were also found at examination. The animal had apparently been attracting other male dogs, as does a bitch in oestrus, for some months, and had been noticed to squat to micturate rather than adopting the usual three-legged stance of the male. The dog was subjected to surgery (anaesthesia, thiopentone Na, cyclopropane/O_2) and a large tumour weighing 38.5 g. was removed from the scrotum and transferred to the laboratory in crushed ice. The tumour, occupying the whole of the left testis, was very hard and of a homogenous creamy-white colour on section.

(b) Histological description.

Most of the tumour was composed of columns and sheets of cells dispersed in an abundant fibro--cellular stroma (Plate 2). In some places, however, there were distorted tubular structures lined by tumour cells and containing a mixture of tumour cells and vacuolated cell debris in their lumina. The neoplastic cells had oval reticulated pale nuclei similar to those of Sertoli cells. In general, their cytoplasm was extremely vacuolated, and the presence of large vacuoles often displaced the nucleus of the cell and distorted its appearance. The mitotic rate was low. The dense fibrous stroma, the nuclear morphology, especially in the cells which are not extremely vacuolated, and the crude tubular structures suggest that this is a Sertoli cell tumour.

(c) Preparation of tissue and conditions of incubation.

The tissue was chopped as described in Method 3 (see Section IV, B iii), and incubated with isotopically-labelled steroids in two vessels.

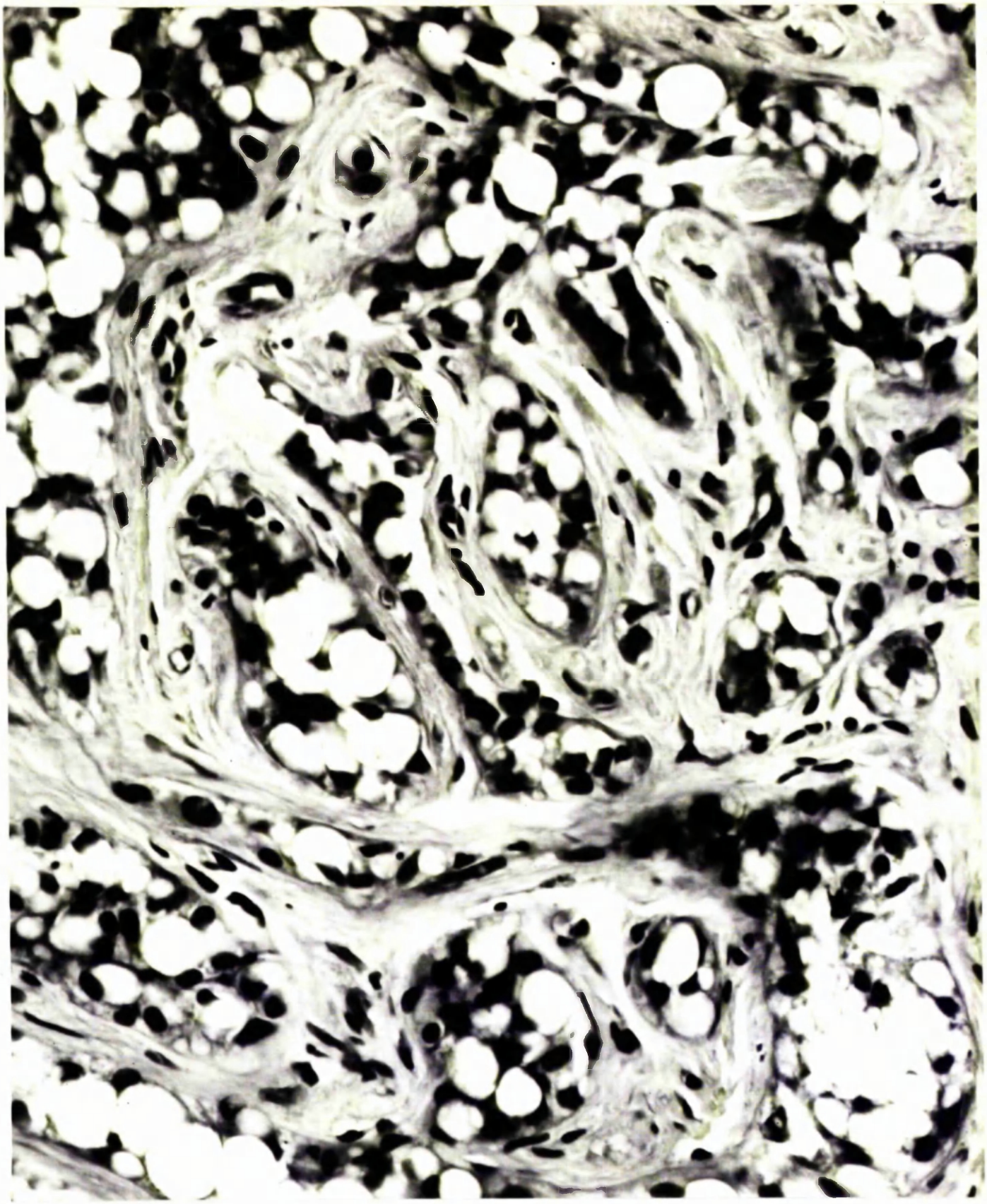


PLATE 2. Sertoli cell tumour (1).

(x 500)

The mince, 2 g. in each case, was suspended in 24 ml. Krebs-Ringer bicarbonate-glucose solution and incubated with (1) 59 mμmole each of 7α-^{3}H pregnenolone (254 μC/μmole) and 4-^{14}C DHA (34 μC/μmole); and with (2) 59 mμmoles each of 7α-^{3}H pregnenolone (254 μC/μmole) and 4-^{14}C testosterone (34 μC/μmole). Incubation was allowed to continue, in each case, for 2.5 hr. at 37° and was stopped by the addition of 10 ml. acetone and cooling to -15°.

The following steroids, 300 μg. in each case, were added; to incubation (1) pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxy-progesterone, DHA, androst-5-ene-diol, androstene-dione, testosterone, DHA sulphate, pregnenolone sulphate, 17α-hydroxypregnenolone sulphate, oestrone, oestradiol-17β and oestriol; and to incubation (2) 16α-hydroxypregnenolone, 16α-hydroxyprogesterone, DHA, androst-5-ene-diol, androstenedione, testosterone, oestrone, oestradiol-17β, oestriol and equilenin.

Extraction Procedure II (see Section IV, D ii) was used to extract and fractionate the steroids from the incubation media.

(d) Investigation of the fractions containing neutral steroids.

The two fractions obtained containing neutral steroids were chromatographed on silica gel $HF_{254/366}$ in solvent system A (Table 4) and the steroids eluted either singly or in pairs depending upon the resolution achieved from the other carriers added. Pregnenolone and DHA were eluted together as were testosterone and 17α-hydroxyprogesterone; androst-5-ene-diol and 17α-hydroxypregnenolone; and 16α-hydroxyprogesterone and 16α-hydroxypregnenolone.

Pregnenolone and DHA were resolved by "double--running" in system A which involves removing the plate after a single run, allowing it to dry and then replacing it in the same solvent system without eluting the steroids. The two steroids, thus separated, were eluted, acetylated and chromatographed in system B. Saponification and chromatography, again in system A, were performed before determining their specific activities. The acetates of each steroid were again prepared and

purified in system B for a second specific activity and the reduction products, pregn-5-ene-3β,20β-diol and androst-5-ene-diol provided further specific activities after chromatography in systems A and C respectively. Testosterone and 17α-hydroxy-progesterone were separated in system D and each subjected to an acetylation reaction. Testosterone acetate was purified in system A, eluted, saponified and re-run in the same system before its specific activity was determined. Oxidation and acetylation provided further derivatives, each of which were run in system A for purification. The unaltered 17α-hydroxyprogesterone from the acetylation reaction was run in system D before its specific activity was measured. Reduction to 17α,20β-dihydroxypregn-4-en-3-one (system C) and acetylation of this product to 17α-hydroxy-20β-acetoxypregn-4--en-3-one (system F) yielded two further derivatives for specific activities.

Androst-5-ene-diol and 17α-hydroxypregnenolone were separated in system C and then acetylated. The products of acetylation were chromatographed in

system E and then saponified before re-running in system C. Specific activities were determined before re-acetylating and chromatographing in system E. Oxidation of androst-5-ene-diol to 6-oxo-androstenedione achieved a third specific activity for ^{14}C for this steroid (see appendix 10) after running in system A. The 16α-hydroxypregnenolone and 16α-hydroxyprogesterone were resolved in system G. Each steroid was eluted separately, acetylated and chromatographed again in system G before measuring their specific activities. The reduced product of 16α-hydroxyprogesterone, 16α,20β--dihydroxypregn-4-en-3-one was run in system G, before measuring its specific activity. The acetate of 16α-hydroxypregnenolone was purified in system A.

Progesterone, eluted singly, was subjected to an acetylation reaction and re-run in system A before determining its specific activity. Further measurements were made after reduction to 20β-hydroxy-pregn-4-en-3-one and acetylation of this product to 20β-acetoxypregn-4-en-3-one, both of which derivatives were purified in system A. Androstenedione was also subjected to an acetylation reaction before

re-running in system A. Reduction to testosterone and acetylation of this product to testosterone acetate, both of which were chromatographed in system A, provided further derivatives for specific activity measurements.

(e) Investigation of the fractions containing phenolic steroids.

The fractions containing phenolic steroids were chromatographed initially in solvent system H and all eluted singly except oestrone and equilenin from the second incubation which ran so closely together that they were removed together.

Oestrone and equilenin were resolved by "double-running" (see Section IV, L iii, d) in system O and after elution each steroid was further purified by chromatography in system M. A specific activity was determined for equilenin at this stage and, as no radioactivity was found, no further investigations were undertaken with this steroid. The oestrone was acetylated and chromatographed in system M, eluted, saponified and re-run in system H before specific activity determination. The methyl

ether was prepared and purified in system M and the reduced product (oestradiol-17β) was purified in system H prior to measurement of specific activities.

Oestradiol-17β was acetylated and chromatographed in system N before saponification and purification in system H. The parent steroid was measured and also the diacetate. The oxidation product, oestrone, was prepared and run in system H and the methyl ether in system L to provide further derivatives.

Oestriol was re-run in system L, eluted and acetylated. The tri-acetate was purified by chromatography in system B and a specific activity determined. The free steroid was recovered after saponification and chromatographed in system L. A methyl-ether was formed from the oestriol from the second incubation and purified in system L.

(f) Investigation of the fractions containing conjugated steroids.

This fraction was dealt with as described in the foregoing experiment (see Section IV. L ii, c).

(g) Results.

The radioactivity isolated in the various fractions from the two incubations and associated with the individual steroids investigated is given in Table 10. The evidence for the radiochemical purity and identity of the steroids investigated may be found in Table 11.

Oxidation of androst-5-ene-diol by Kiliani reagent results in the formation of androst-4-ene--3,6,17-trione (6-oxo-androstenedione). During this procedure, 7α-tritium is lost, presumably by enolisation at the 6,7 position (see appendix 10). Hence, this derivative preparation procedure was not employed in the investigation of tritiated androst--5-ene-diol. The carbon level remains constant, however, if the compound is pure.

It is noteworthy that during the measurement of specific activities of derivatives of oestradiol--17β, the tritium content remained constant during oxidation, acetylation and chromatography, but eventually disappeared after methylation and subsequent chromatography. It is thus assumed for

Table 10. Recovery of radioactivity in the various fractions (dpm x 10^{-3}), and the total radioactivity associated with the steroids isolated from the incubations (dpm x 10^{-3}) with Sertoli cell tumour (1)

Percentages of the initial radioactivity are shown in parentheses

	INCUBATION (1)				INCUBATION (2)			
	From 7α-^{3}H pregnenolone		From 4-^{14}C DHA		From 7α-^{3}H pregnenolone		From 4-^{14}C testosterone	
Neutral steroid fraction	32,090	(83.9)	5,182	(84.3)	34,330	(89.8)	4,783	(91.8)
pregnenolone	32,827	(85.8)	0		*		*	
17α-OH pregnenolone	484.3	(1.27)	0		*		*	
16α-OH pregnenolone	*		*		110.6	(0.29)	0	
progesterone	643	(1.68)	0		*		*	
17α-OH progesterone	0		0		*		*	
16α-OH progesterone	*		*		0		0	
DHA	0		2,596	(42.2)	14.1	(0.04)	1.9	(0.04)
androst-5-ene-diol	398.3	(1.04)	82.4	(1.34)	793.1	(2.07)	0	
androstenedione	0		2,345	(38.2)	0		715.4	(13.7)
testosterone	18.0	(0.05)	373.0	(6.06)	0		4,460	(85.6)
Phenolic steroid fraction	898.7	(2.35)	200.2	(3.26)	265.6	(0.69)	65.0	(1.25)
oestrone	0		18.5	(0.30)	0		7.5	(0.14)
oestradiol-17β	0		10.2	(0.17)	0		28.0	(0.54)
oestriol	0		0		0		0	
equilenin	*		*		0		0	
Conjugated steroid fraction	629.2	(1.65)	62.0	(1.01)	*		*	
pregnenolone sulphate	274.5	(0.72)	0		*		*	
17α-OH pregnenolone sulphate	5.9	(0.02)	0		*		*	
DHA sulphate	7.8	(0.02)	11.3	(0.18)	*		*	

* not investigated.

Table 11. Evidence for the identity of the steroids isolated from the incubations with Sertoli cell tumour (1)

	Material investigated and chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	SPECTIFIC ACTIVITIES (dpm/mμmole) INCUBATION (1) ^{3}H	INCUBATION (1) ^{14}C	INCUBATION (2) ^{3}H	INCUBATION (2) ^{14}C
	Neutral steroids						
1.	pregnenolone						
	-	pregnenolone	A	34,100	0	*	*
	acetylation	pregnenolone acetate	B	33,100	0	*	*
	reduction	pregn-5-ene-3β,20β-diol	A	36,500	0	*	*
2.	17α-OH pregnenolone						
	-	17α-OH pregnenolone	C	510	0	*	*
	acetylation	17α-OH pregnenolone acetate	E	560	0	*	*
3.	progesterone						
	-	progesterone	A	690	0	*	*
	reduction	20β-hydroxypregn-4-en-3-one	A	680	0	*	*
	acetylation	20β-acetoxypregn-4-en-3-one	A	650	0	*	*
4.	17α-OH progesterone						
	-	17α-OH progesterone	D	0	0	*	*
	reduction	17α,20β-dihydroxypregn-4-en-3-one	C	0	0	*	*
	acetylation	17α-hydroxy-20β-acetoxypregn-4-en-3-one	F	0	0	*	*
5.	16α-OH pregnenolone						
	-	16α-OH pregnenolone	G	*	*	180.9	0
	acetylation	3β,16α-diacetoxypregn-5-en-20-one	A	*	*	185.8	0
6.	16α-OH progesterone						
	-	16α-OH progesterone	G	*	*	0	0
	acetylation	16α-acetoxypregn-4-ene-3,20-dione	A	*	*	0	0
	reduction	16α,20β-dihydroxypregn-4-en-3-one	G	*	*	0	0
7.	androst-5-ene-diol						
	-	androst-5-ene-diol	C	405	77.2	761.5	0
	acetylation	androst-5-ene-diol diacetate	E	364	81.2	771.9	0
	oxidation	6-oxo-androstenedione	A	‡	80.8	*	*
8.	DHA						
	-	DHA	A	0	2,510	13.6	2.3
	acetylation	DHA acetate	B	0	2,510	13.8	2.2
	reduction	androst-5-ene-diol	A	0	2,460	13.1	1.0
9.	androstenedione						
	-	androstenedione	A	0	2,156	0	659.0
	reduction	testosterone	A	0	2,202	0	684.0
	acetylation	testosterone acetate	A	0	2,352	0	702.0
10.	testosterone						
	-	testosterone	A	17.7	370	0	4,354.5
	acetylation	testosterone acetate	A	16.1	356	0	4,318.5
	oxidation	androstenedione	A	18.0	350	0	4,170.7

Table 11 (Continued). Sertoli cell tumour (1).

	Material investigated and chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	SPECIFIC ACTIVITIES (dpm/mμmole) INCUBATION (1) ^{3}H	INCUBATION (1) ^{14}C	INCUBATION (2) ^{3}H	INCUBATION (2) ^{14}C
	Phenolic fraction						
1.	oestrone						
	-	oestrone	H	0.24	16.24	0	6.63
	methylation	oestrone-3-methyl ether	M	0.67	17.12	0	6.85
	reduction	oestradiol-17β	H	*	*	0	6.72
	acetylation	oestrone acetate	M	0	18.62	*	*
2.	oestradiol-17β						
	-	oestradiol-17β	H	34.2	9.62	45.19	25.35
	acetylation	oestradiol-17β-diacetate	M	*	*	46.02	24.37
	methylation	oestradiol-17β-3-methyl ether	L	0	9.60	0	26.44
	oxidation	oestrone	H	12.73	8.46	13.53	26.99
3.	oestriol						
	-	oestriol	L	0	0	0	0
	methylation	oestriol-3-methyl ether	L	0	0	0	0
	acetylation	oestriol triacetate	B	*	*	0	0
4.	equilenin						
	-	equilenin	H	*	*	0	0
	Steroid sulphates						
1.	pregnenolone sulphate						
	-	pregnenolone sulphate	P	‡	‡	*	*
	solvolysis	pregnenolone	A	379	0	*	*
	acetylation	pregnenolone acetate	B	386	0	*	*
2.	17α-OH pregnenolone sulphate						
	-	17α-OH pregnenolone sulphate		*	*	*	*
	solvolysis	17α-OH pregnenolone	C	8.08	0	*	*
	acetylation	17α-OH pregnenolone acetate	E	8.98	0	*	*
3.	DHA sulphate						
	-	DHA sulphate	J	‡	‡	*	*
	solvolysis	DHA	A	9.45	15.06	*	*
	acetylation	DHA acetate	B	11.39	14.80	*	*
	reduction	androst-5-ene-diol	A	9.65	14.38	*	*

* not measured. ‡ unsatisfactory result.

the present from these experimental results that this radioactivity is not associated with the oestradiol--17β. There was insufficient material remaining to investigate the loss of radioactivity at the methylation stage. The results for the tritium isolated with oestradiol-17β have, however, been included in Table 11.

It is perhaps worthy of note that in neither incubation was tritium from 7α-^{3}H pregnenolone isolated in the co-incubated C_{19}-steroid, viz. DHA (incubation 1) and testosterone (incubation 2). These two steroids were, however, isolated with the tritium label when they themselves were not being used as substrates. This would suggest a suppressive effect of the steroids on their own formation and obviating itself, in this instance, because of the low level of metabolism of pregnenolone by the tumour tissue (i.e. 14%) and the very small conversion to these particular steroids that would have been realised (DHA - 0.04% and testosterone - 0.05%) but, apparently, for their presence in even small amounts (approx. 25 μg.) as isotopically-labelled substrates.

Neher & Kahnt (1965) during their studies on the selective inhibition of various steps in the biosynthesis of steroids by rat testicular tissue showed that the degradation of 4-^{14}C 17α-hydroxy-progesterone was partially inhibited by inactive substrate at a steroid to tissue ratio of 3:10,000 and also by progesterone and 20β-hydroxypregn-4--en-3-one at ratios of 1:10,000 and 8:100,000 respectively. They found complete inhibition of the degradation of 7α-^{3}H 17α-hydroxypregnenolone by the addition of pregnenolone or progesterone to steroid to tissue ratios of 3:10,000. Testosterone, however, had no effect at a ratio of up to 3:10,000.

The steroid to tissue ratios of the incubations reported here, with respect to DHA and testosterone, were, approximately, 8:1,000,000 indicating the sensitivity of the enzymes of this neoplasm to even very low steroid concentrations.

There have been reports on the inhibition of formation of adrenocortical hormones (Birmingham & Kurlents, 1958; Peron, Moncloa & Dorfman, 1960; Fukui, Takeuchi, Watanabe, Kumagai, Sano & Nishino, 1961; Fekete & Görög, 1963) by the addition of

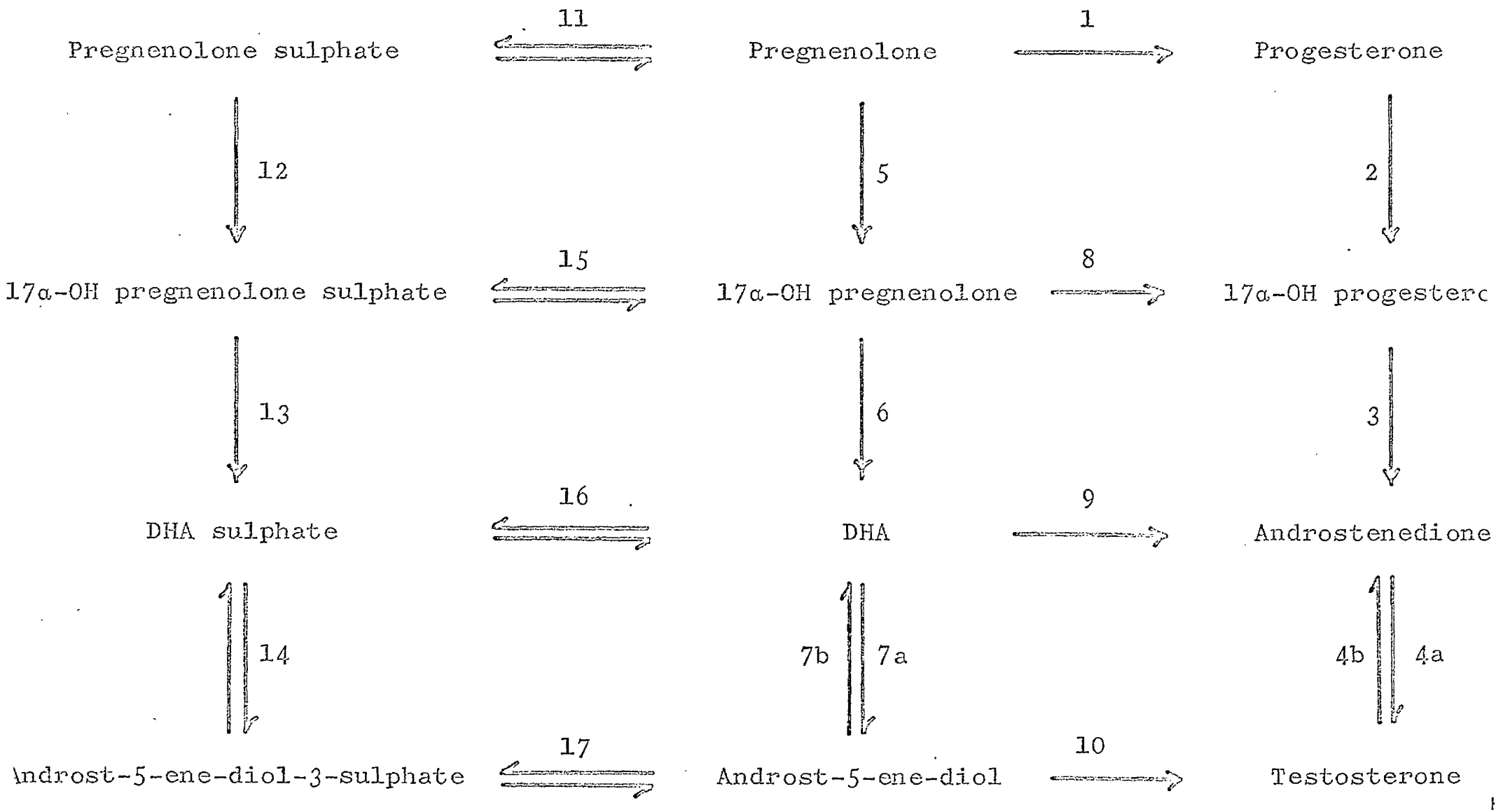
various corticosteroids or adrenal extracts to incubations of adrenal tissues or by their administration to the living animal, providing evidence for a "feed-back" mechanism at the adrenal level.

It is for consideration whether a similar "feed-back" mechanism is operative in the testes, and in a neoplasm such as the type described here, where certain enzyme systems are functioning at levels much lower than normal, the thresholds of inhibition are more easily surpassed.

It may be seen from Table 10 that 0.04% of the radioactivity from the incubated 4-^{14}C testosterone is associated with the DHA isolated. It was found not possible to lose this label from DHA after several chromatographic procedures and the formation of various derivatives, as shown in Table 11. The evidence remains, therefore, that a reversal of the 3β-hydroxysteroid dehydrogenase-isomerase enzyme system has taken place (reaction 10, Text fig. 7) followed by dehydrogenation of androst-5-ene-diol at the 17-position (reaction 7b) to form DHA.

Text figure 7.

Possible pathways for steroid biosynthesis in the dog testis.



Conversely with regard to the formation of androstenedione (14%) from testosterone, reversal of the 3β--hydroxysteroid dehydrogenase-isomerase indicated by reaction 9 would convert this compound directly to DHA as described by Ward & Engel (1964, 1966). Rosner, Hall & Eik-Nes (1965) have observed the analogous reduction of progesterone to pregnenolone (reversal of reaction 1, Text fig. 7) by homogenates of rabbit testes. If, as Ward & Engel (1966) suggest, the displacement of the equilibrium of this reversible system can be caused by one of the products of the system, then, in the presence of DPNH, testosterone and androstenedione could be converted to their respective homoallylic alcohols.

The other results of these incubations will be discussed more fully in the light of the findings from the incubations with normal testicular tissue described in the next section.

(iv) Normal testicular tissue (2).

An investigation of the metabolism, in vitro, of 7α-^{3}H pregnenolone and 4-^{14}C DHA by normal canine testicular tissue.

(a) Preparation of tissue, incubation procedure and method of extraction of steroids.

The tissue was obtained from a dog being destroyed after a road accident. There were no clinical signs of endocrinological abnormalities and the histological picture of the tissue was normal.

The tissue was prepared and incubated in precisely the same manner and with the same amounts of radioactive steroids as Incubation (1) of the Sertoli cell tumour (1) investigation (see Section IV, L iii), i.e. 2 g. minced tissue in 24 ml. Krebs--Ringer bicarbonate glucose solution for 2.5 hr. at 37^{o} with 59 mμmoles each of 7α-^{3}H pregnenolone (254 μC/μmole) and 4-^{14}C DHA (34 μC/μmole).

Oestradiol-17α was added in addition to those steroids investigated in the previous incubations.

The steroids were extracted, using Procedure II (Section IV, D ii), and the various fractions examined as for the preceding Sertoli cell tumour experiment.

(b) Results.

The radioactive content of the three fractions obtained from this incubation and total radioactivity associated with each of the steroids investigated are given in Table 12. The evidence for the radiochemical purity and identity of the steroids isolated is shown in Table 13.

In agreement with the last experiment the oestradiol-17β appeared to be tritiated until methylation. These results are shown in Table 13.

(c) Discussion.

In order to assess more fully the enzymic derangement apparent from the investigations of the Sertoli cell tumour of Section IV, L iii, the results of that experiment and the one described above with normal testicular tissue are discussed together.

Table 12. Normal testicular tissue (2).

Recovery of radioactivity in the various steroid fractions (dpm x 10^{-3}), and the total radioactivity associated with the individual steroids isolated from the incubation (dpm x 10^{-3})

Percentages of the radioactivity initially present are shown in parentheses

	From 7α-^{3}H pregnenolone		From 4-^{14}C DHA	
Neutral steroid fraction	35,142	(91.9)	3,976.5	(64.7)
pregnenolone	11,797	(30.9)	0	
17α-OH pregnenolone	1,966.1	(5.14)	0	
16α-OH pregnenolone	110.4	(0.29)	0	
progesterone	218.5	(0.57)	0	
17α-OH progesterone	435.3	(1.14)	0	
16α-OH progesterone	0		0	
DHA	382	(1.00)	2,163.2	(35.2)
androst-5-ene-diol	3,589.7	(9.39)	1,178.3	(19.2)
androstenedione	889.7	(2.33)	345.4	(5.62)
testosterone	3,595.6	(9.40)	1,613.1	(26.2)
Phenolic steroid fraction	841.8	(2.20)	100.2	(1.63)
oestrone	0		0.4	(<0.01)
oestradiol-17β	0.45	(<0.01)	0.4	(<0.01)
oestradiol-17α	2.5	(<0.01)	0	
oestriol	0		0	
Conjugated steroid fraction	1,029.3	(2.69)	58.5	(0.95)
pregnenolone sulphate	30.3	(0.08)	0	
17α-OH pregnenolone sulphate	0		0	
DHA sulphate	5.9	(0.02)	8.1	(0.13)

Table 13. Evidence for the identity of the steroids isolated from the incubation with normal testicular tissue (2).

Material investigated and the chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	Specific activities (dpm/mμmole) ^{3}H	^{14}C
Neutral steroids				
1. pregnenolone				
-	pregnenolone	A	12,556.4	0
acetylation	pregnenolone acetate	B	12,836.1	0
reduction	pregn-5-ene-3β,20β-diol	A	11,885.8	0
2. 17α-OH pregnenolone				
-	17α-OH pregnenolone	C	2,133.7	0
acetylation	17α-OH pregnenolone acetate	E	2,217.8	0
3. 16α-OH pregnenolone				
-	16α-OH pregnenolone	G	194.8	0
acetylation and saponification	16α-OH pregnenolone	G	171.5	0
4. progesterone				
-	progesterone	A	223.5	0
reduction	20β-hydroxypregn-4-en-3-one	A	233.7	0
acetylation	20β-acetoxypregn-4-en-3-one	A	230.0	0
5. 17α-OH progesterone				
-	17α-OH progesterone	D	455.6	0
reduction	17α,20β-dihydroxypregn-4-en-3-one	C	495.2	0
acetylation	17α-hydroxy-20β-acetoxypregn-4-en-3-one	F	487.9	0
6. 16α-OH progesterone				
-	16α-OH progesterone	G	0	0
acetylation	16α-acetoxypregn-4-ene-3,20-dione	G	0	0
reduction	16α,20β-dihydroxypregn-4-en-3-one	G	0	0
7. DHA				
-	DHA	A	364.5	2,127.0
acetylation	DHA acetate	B	385.9	2,018.0
reduction	androst-5-ene-diol	A	348.7	2,085.0
8. androst-5-ene-diol				
-	androst-5-ene-diol	C	3,530.2	1,161.7
acetylation	androst-5-ene-diol diacetate	E	3,410.4	1,116.4
9. androstenedione				
-	androstenedione	A	863.7	323.3
reduction	testosterone	A	866.4	336.4
acetylation	testosterone acetate	A	814.5	328.1
10. testosterone				
-	testosterone	A	3,501.8	1,541.4
acetylation	testosterone acetate	A	3,303.0	1,502.5
oxidation	androstenedione	A	3,550.5	1,601.8

Table 13 (Continued). Formal testicular tissue (2).

Material investigated and the chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	Specific activities (dpm/mμmole) ^{3}H	^{14}C
Phenolic steroids				
1. oestrone				
-	oestrone	H	0	0.21
acetylation	oestrone acetate	M	0	0.39
methylation	oestrone-3-methyl ether	M	0	0.43
2. oestradiol-17β				
-	oestradiol-17β	H	7.81	0.27
acetylation	oestradiol-17β-diacetate	M	7.38	0.50
oxidation	oestrone	H	7.07	0.34
methylation	oestradiol-17β-3-methyl ether	L	0.41	0.24
3. oestradiol-17α				
-	oestradiol-17α	H	2.46	0
acetylation	oestradiol-17α-diacetate	M	2.04	0
4. oestriol				
methylation	oestriol-3-methyl ether	L	0	0
Steroid sulphates				
1. pregnenolone sulphate				
-	pregnenolone sulphate	P	42.99	0
solvolysis	pregnenolone	A	*	*
acetylation	pregnenolone acetate	B	41.50	0
2. 17α-OH pregnenolone sulphate				
-	17α-OH pregnenolone sulphate		*	*
solvolysis	17α-OH pregnenolone	C	*	*
acetylation	17α-OH pregnenolone acetate	E	0	0
3. DHA sulphate				
-	DHA sulphate	J	7.93	10.98
solvolysis	DHA	A	‡	9.89
acetylation	DHA acetate	B	7.32	10.52

* not investigated.

‡ unsatisfactory result.

The conversion figures from the three incubations have been brought together in Table 14 for ease of comparison.

The Sertoli cell tumour of the canine testis has aroused considerable interest for many years due to the high incidence of feminization and other signs of endocrine dysfunction often associated with it (see the Introduction, Section III). A great deal of clinical evidence has provided tentative support for the hypothesis that this type of tumour is a source of oestrogenic hormones. Wooldridge (1908) described a Pomeranian, which for 1 yr. had shown a gradual enlargement of the right testis with a simultaneous loss of hair. Prominence of the penile sheath could be seen from the photographs. The unusual fact was that Wooldridge considered the tumour to have no relationship with the other clinical signs.

Testicular tumours associated with mammary, prostatic and other changes in three cryptorchid dogs were reported by Greulich & Burford (1936). Subsequent communications (Zuckerman & Groome, 1937; Arnold, Hamperl, Holtz, Junkmann & Marx, 1937; Huggins & Clark, 1940; Gardner & De Vita, 1940;

Mulligan, 1943) indicated that all the changes occurring in the various tissues of dogs affected by this type of testicular tumour could be mimicked by the administration of oestrogens; oestrone, oestradiol benzoate, oestradiol monobenzoate and diethylstilboestrol were all used. Mulligan (1944) summarised the situation at the time: "The similarity between the changes produced by estrogens and the several features of this symptom-complex, suggests that the carcinoma of the testis elaborates estrogenic material or at least a substance altered in some metabolic process to a compound having a feminizing action". A more complete list of authors working on this subject and the various effects evoked by oestrogens is provided by Jabara (1962) in her paper on the tissue changes in the dog following stilboestrol administration.

The presence of an oestrogenic substance in these tumours was demonstrated by Huggins & Moulder (1945). They extracted 70 μg. of a substance described as α-oestradiol from 1 Kg. of tumour tissue removed from dogs showing clinical evidence of feminization. Several years later Brodey &

Martin (1958) provided evidence by bioassay for the presence in the urine from affected dogs of oestrogenic material, the amount of which dropped emphatically after surgical removal of the tumour. The case reported by Berthrong, Goodwin & Scott (1949) presented with generalized alopecia, apparent pruritis and marked pigmentation of the abdominal and scrotal skin. The posterior teats were enlarged and other dogs found the patient sexually attractive. Urine analysis suggested high oestrogen and low 17-oxosteroid excretion. These authors did not describe, however, the techniques they employed in this analysis and therefore too much reliance should not be placed on these results. The right testis was almost completely replaced by a tumour weighing 45 g. and described as bearing resemblance to a Sertoli cell tumour. Metastases were found. These authors concluded that the testis produced an oestrogenic hormone which originated in the Sertoli cells.

Steroid biosynthesis by such a tumour has not, hitherto, been described and the results reported here provide interesting information on the steroido-

genic ability of this tissue when compared with that of normal testicular tissue from another adult dog. During the discussion, reference will be made essentially to Table 14, in which the radioactivity (as percentages of the radioactivity initially incubated) isolated with the steroids investigated from the Sertoli cell tumour (1) incubations (1) and (2) and the normal testis incubation have been drawn together, and to Text figure 7. The numbers in the diagram refer to particular enzymes and the reactions they catalyze at those particular points in the biosynthetic sequence, and will be referred to as such in the text.

From the diagram (Text fig. 7) there are at least seventeen possible reactions in numerous pathways involved in transforming pregnenolone to testosterone. Although progesterone and 17α--hydroxypregnenolone were formed by the tumour via reactions (1) and (5) respectively, no tritiated 17α-hydroxyprogesterone was found. A high rate of metabolism of this compound would surely have resulted in the formation of tritiated androstenedione

and/or testosterone via reactions (3) and (4a) but as only a trace of one of these probable metabolites (0.05% testosterone) was found, it is concluded that defective formation of 17α-hydroxy-progesterone rather than its rapid utilization was the reason for its absence. This would indicate an impairment of amount or activity of progesterone--17α-hydroxylase (reaction 2) and 17α-hydroxy-pregnenolone 3β-hydroxydehydrogenase-isomerase (reaction 8). The fact that it was possible to demonstrate 17α-hydroxylation of pregnenolone in the same experiment (reaction 5) provides some evidence to support the view that steroid specific 17α-hydroxylases exist in testicular tissue. A similar observation has been made for ovarian tissue (Griffiths, Grant, Browning, Cunningham & Barr, 1966). It could be argued that 17α-hydroxylation of progesterone would have been demonstrated were the latter compound produced in greater amounts and that the different conversion figures for the two 17α--hydroxylated steroids was due to the greater relative amount of pregnenolone to progesterone that was presented to the tissue. It may be seen from

Table 14 that the combined conversion figures of progesterone and 17α-hydroxyprogesterone in the tumour is 1.68% and in the normal testis 1.71%. The difference being that in the former this figure is made up solely from the progesterone while in the normal testis 1.14% is due to the 17α-hydroxy-progesterone, indicating dysfunction of progesterone--17α-hydroxylase activity in the Sertoli cell tumour.

While the 17α-hydroxypregnenolone-3β-hydroxy-dehydrogenase-isomerase (reaction 8) is impaired or absent the results from the carbon-labelled steroids show that DHA-3β-hydroxydehydrogenase-isomerase (reaction 9) is active in the neoplasm. This provides evidence for specific 3β-hydroxysteroid dehydrogenase in the testis. Such specific enzymes have already been shown to exist in adrenal tissue (Weliky & Engel, 1963).

The relative abilities of the normal and neoplastic tissues to metabolize the steroid precursors with which they were presented show interesting differences. DHA is metabolized in similar quantitative manner by normal (65%) and neoplastic (58%) tissues. Androst-5-ene-diol (19%)

Table 14. To compare the relative amounts of radioactivity isolated with the various steroids investigated in the incubations with the Sertoli cell tumour and the normal dog testis. The figures are percentages of the initial radioactivity.

	Sertoli cell tumour (1)			Normal testis (2)	
	From Pregnenolone	From DHA	From Testosterone	From Pregnenolone	From DHA
pregnenolone	85.8	0		30.9	0
17α-OH pregnenolone	1.27	0		5.14	0
16α-OH pregnenolone	0.29*	‡		0.29	0
progesterone	1.68	0		0.57	0
17α-OH progesterone	0	0		1.14	0
16α-OH progesterone	0*	‡		0	0
DHA	0	42.2	0.04	1.00	35.2
androst-5-ene-diol	1.04	1.34	0	9.39	19.2
androstenedione	0	38.2	13.7	2.33	5.62
testosterone	0.05	6.06	85.6	9.40	26.2
oestrone	0	0.30	0.14	0	<0.01
oestradiol-17β	0	0.17	0.54	<0.01	<0.01
oestradiol-17α	‡	‡	‡	<0.01	0
oestriol	0	0	0	0	0
equilenin	0*	‡	0	‡	‡
pregnenolone sulphate	0.72	0		0.08	0
17α-OH pregnenolone sulphate	0.02	0		0	0
DHA sulphate	0.02	0.18		0.02	0.13

* formed from 7α-^{3}H pregnenolone in the second incubation with tumour tissue.

‡ not examined.

and testosterone (26%) are the main metabolites from the normal testis, whereas androstenedione (38%) predominates in the tumour incubation in which much less testosterone (6%) is formed. This points to an impairment in reduction at the 17-position (reaction 4a), which might be due to decreased enzyme synthesis or activity. The testosterone-17β-hydroxydehydrogenase (reaction 4b) was functioning well in that 4-^{14}C testosterone was converted, in reasonable yield, to androstenedione (14%) in the second tumour incubation. Slaunwhite, Sandberg, Jackson & Staubitz (1962) demonstrated an inhibition of reduction of androstenedione to testosterone in homogenates of testicular tissue from men with prostatic carcinoma who had been receiving parenteral diethyl stilboestrol. The ability of the tumour tissue to metabolize pregnenolone (14%) is very much less than that of the normal testis (69%). It is for consideration whether the oestrogens produced by the tumour could have influenced dehydrogenase and desmolase activities in this tissue in the light of the work of Samuels, Short & Huseby (1964) who reported a suppression of steroid 17α-hydroxylase and

pregnene-17,20-lyase in mouse testicular tissue after oestrogen administration.

The decreased 17α-hydroxylation of both pregnenolone (reaction 5) and progesterone (reaction 2) in the tumour may well have resulted from anoxia of the tumour tissue related to its size, fibrosity and rate of growth. It is well known that steroid hydroxylation requires molecular oxygen, and Nakano, Sato & Tamaoki (1966) have produced evidence for the requirement of molecular oxygen for the conversion of 17α-hydroxyprogesterone to androstenedione (reaction 3). It is interesting, therefore, to speculate on the possibility that oxygen lack may impair the activity or amount of these biosynthetic enzymes in vivo, the effects of which persisting in vitro.

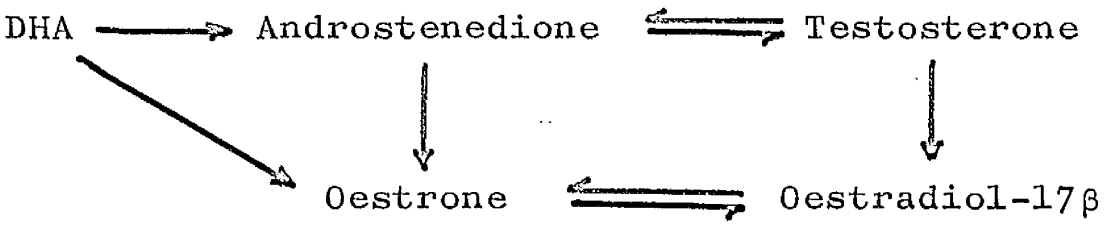
Evidence is presented for the presence of a 16α-hydroxylase by the formation of 16α-hydroxy-pregnenolone (0.29%) in both normal and neoplastic tissues although the 16α-hydroxyprogesterone isolated was unlabelled. It has been suggested already with regard to the formation of oestriol from pregnenolone by the human tumour (see Section IV,

L i h) that perhaps an important intermediate could be 16α-hydroxyprogesterone. The results from these investigations give no indication as to the part that 16α-hydroxypregnenolone may play in steroid synthesis in the canine testis, as oestriol was not formed in any one of the three incubations described above with canine testicular tissue. There is presented by these results, however, once more, an example of steroid specific enzymes; both normal and neoplastic tissues possessing the pregnenolone--16α-hydroxylase but not progesterone-16α-hydroxylase. Any question of inability of progesterone to enter cells as compared with pregnenolone is discounted by the formation of 17α-hydroxyprogesterone in the normal testis incubation.

The formation of oestrone and oestradiol-17β from both DHA and testosterone by the Sertoli cell tumour is of particular interest in that it shows for the first time the ability of this tissue to form oestrogens. Very little of these two steroids was formed from DHA by the normal dog testis; the conversions are recorded as less than 0.01%. It is of interest to speculate on the immediate

precursors of these oestrogens in the tumour. Oestrone predominated over oestradiol-17β by a ratio of 1.8:1 when DHA was incubated and androstenedione was the chief metabolite (38%). In the incubation with 4-^{14}C testosterone it was found that oestradiol--17β predominated by a ratio of 3.9:1 over oestrone, with androstenedione being, again, the chief metabolite but at a much lower conversion (14%). It is suggested that the tumour tissue is able to convert both androstenedione and testosterone to oestrone and oestradiol-17β respectively and although it is recognised that oestrone and oestradiol-17β are freely interconvertible, their relative production in this tissue depends upon the immediately predominating C_{19} steroid.

Thus



The sulphates of pregnenolone, 17α-hydroxy-pregnenolone and DHA were formed again by the tumour

as in the case of the human interstitial cell tumour. Pregnenolone sulphate and DHA sulphate were formed by the normal dog testis in slightly less amounts. These results do not aid the development of the hypothesis formulated after the experiment with the human tumour that sulphation of neutral steroids may be connected with the synthesis of oestrogens.

It is considered that the results provided by this in vitro investigation of a representative portion of this neoplasm indicate the steroid biosynthetic activity of the tumour as a whole. It cannot, however, be categorically stated that the biosynthesis is the property, wholly or in part, of the Sertoli cells. Histological examination revealed no Leydig cells, and the interstitial cells that were present were considered to be inactive fibroblasts having no endocrine function (Professor T. Symington, personal communication). If there were, however, to be pockets of these cells within the tumour mass, and not represented on the histological section, their metabolic activity could have contributed to the biosynthetic pattern observed. The location of such Leydig cells in the abnormal

environment provided by the neoplastic Sertoli cells may so alter their metabolic function that the atypical testicular steroid pattern demonstrated could result. Camin, Dorfman, McDonald & Rosenthal (1960) and Schmidt & Tonutti (1956) commented on the effect of virilizing interstitial cell tumours of the testis on adjacent testicular tissue and on the opposite testis. They suggested a local diffusion effect of androgenic substance secreted by the tumour on the surrounding testicular substance and a systemic effect on the opposite organ. The tumour investigated here has been shown to synthesize oestrogens and comment has already been made on the effect that these substances might have on the testicular enzymes.

The evidence remains, however, that the biosynthetic pattern observed is due to the metabolism of the radioactive steroid precursors by the neoplastic Sertoli cells and thus provides good evidence for an endocrine function of these cells within the testis.

It is of considerable interest from the comparative standpoint that a human Sertoli cell tumour investigated

by Dr. R.S. Stempfel (quoted by Lacy, 1962), should have synthesized 17α-hydroxyprogesterone and that the metabolite of this steroid, 5β-pregnane-3α,17α,20α--triol, was secreted in large quantities in the urine. Oestrogens were not shown to be elaborated by this tissue. Griffiths et al. (1963) reported the conversion of progesterone to 17α-hydroxyprogesterone by a Sertoli cell tumour removed from a human male pseudohermaphrodite. Failure to demonstrate oestrogen-synthesis was again reported.

It is for consideration whether these differences in the steroid-metabolism of canine and human neoplastic Sertoli cells may be of significance in relation to their relative tendencies to malignancy.

The investigations discussed above have, in part, been reported (Pierrepoint, Griffiths & Grant, 1966).

(v) Sertoli cell tumour (2).

An investigation of the metabolism, in vitro, of 4-^{14}C DHA and 7α-^{3}H DHA sulphate by a Sertoli cell tumour of the testis from a dog with alopecia and apparent pruritis but with no signs of feminization.

(a) Clinical data.

A Cairn terrier was presented for veterinary examination, having suffered mild alopecia and apparent pruritis for 6 mth. A swelling had been noted in the scrotum for 1 yr. No clinical evidence was ascertained for signs of feminization. A bilateral orchidectomy was performed under general anaesthesia (intravenous thiopentone Na./ /cyclopropane/O_2). The neoplastic testis weighed 70.3 g. and the contralateral organ 3.3 g. The normal testis used in the time-controlled experiment (see Section IV, L ii) weighed 18.5 g., thus indicating the atrophy of the non-neoplastic gland. The tumour was transferred to the laboratory in crushed ice.

(b) Histological description.

The tumour was composed of tubular formations of Sertoli cells interspersed in a well developed fibrous stroma (Plate 3). The neoplastic cells were of typical Sertoli appearance, having large oval reticulated nuclei with clearly defined nuclear membranes containing one or two prominent nucleoli and abundant pale-staining cytoplasm with indistinct borders. The cells were arranged with their long axes extending radially into the tubules and their nuclei situated at a distance from the basement membrane. The structure of the cells was fairly uniform throughout the tumour, though occasional bizarre forms were observed. The mitotic rate was low. The size of the tubules varied considerably. Often there was no central lumen, the tumour cells being several layers deep. When a cavity was present, it frequently contained eosinophilic colloid-like material. The tubules were always well circumscribed with no evidence of invasive activity. The appearance was that of a Sertoli cell tumour.

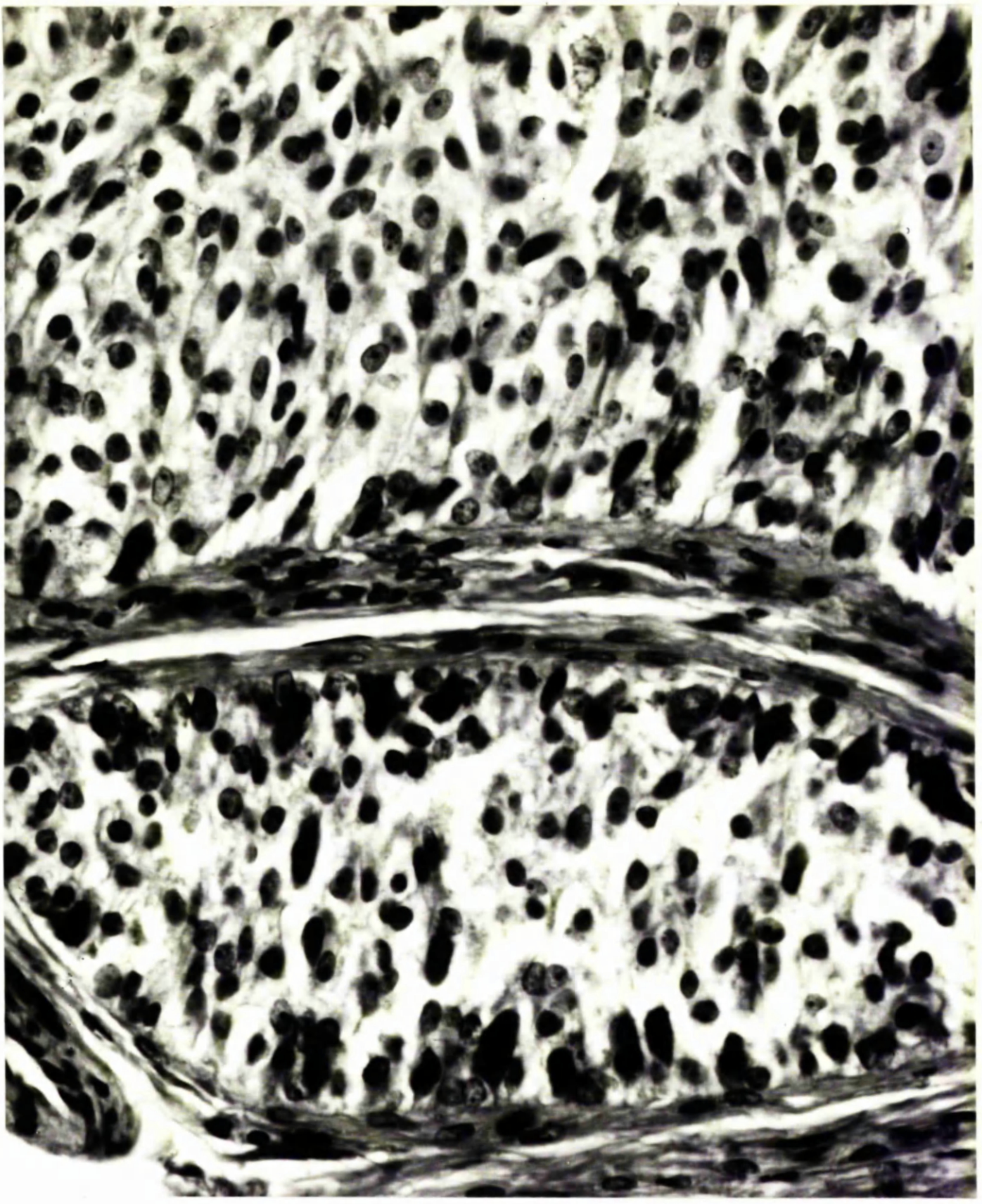


PLATE 3. Sertoli cell tumour (2).

(x 500)

(c) Preparation of tissue and conditions of incubation.

The tissue was prepared as a mince by Method C (see Section IV, B iii) and incubated in the same way as described for the previous dog tissue experiments. In all respects the experimental procedures are the same as for the 2.5 hr. timed experiment (see Section IV, L ii), the results of which will be used for comparative purposes. The minced or chopped tissue (2 g.) was suspended in 24 ml. Krebs-Ringer bicarbonate-glucose solution and incubated with 72.7 mμmoles each of 7-^{3}H DHA sulphate (206.3 μC/μmole) and 4-^{14}C DHA (27.5 μC/ /μmole) for 2.5 hr. at 37°. The reaction was stopped by the addition of 10 ml. acetone and cooling to -15°.

The following steroids were added as cold carriers, 300 μg. of each; DHA, androst-5-ene-diol, androstenedione, testosterone, 11β-hydroxyandrostene-dione, DHA sulphate, oestrone, oestradiol-17β and oestriol. At a later stage 120 μg. testosterone sulphate and free androst-5-ene-diol were added to

the sulphate fraction.

Extraction Procedure II (see Section IV, D ii) was used to extract and fractionate the steroids from the incubation media.

(d) Investigation of the various steroid fractions.

The steroids from the neutral and conjugated steroid fractions were isolated and derivatives formed as described for the experiment with normal canine testicular tissue (see Section IV, L ii). The 11β-hydroxyandrostenedione, after elution from the first chromatographic separation of the neutral steroids, was re-run in system C after which it was subjected to an acetylation reaction and purified in system Q. The eluted steroid was oxidized to adrenosterone and chromatographed in system R. A specific activity was determined before reduction to 11-oxo-testosterone, purifying in system S and then acetylating. The 11-oxo-testosterone acetate was run in system T before measuring its radioactive content.

The phenolic fraction was dealt with as described

in the Sertoli cell tumour (1) investigation (see Section IV, L iii e).

(e) Results.

Table 15 shows the recovery of radioactivity in the three fractions achieved by the extraction procedure and also the total amount of radioactivity associated with each of the steroids investigated. Table 16 gives the evidence, on constancy of specific activities and chromatographic mobility, of the steroids investigated. The percentage conversion achieved with this tissue have been drawn together with those from the 2.5 hr. incubation of normal dog testicular tissue (see Section IV, L ii) in Table 17 for comparative purposes. The results achieved for the sulphates of androst-5-ene-diol and testosterone are uncorrected for losses in extraction and purification as these two steroids were added later.

(f) Discussion.

With reference to Table 17, it may be seen that

Table 15. Recovery of radioactivity in the various steroid fractions and the total radioactivity associated with individual steroids isolated from the incubation (dpm x 10^{-3}). Percentages of the initial radioactivity are shown in parentheses.

	Sertoli cell tumour (2)			
	From 7α-^{3}H DHA sulphate		From 4-^{14}C DHA	
Neutral steroid fraction	2,545.5	(8.97)	4,743.8	(77.2)
DHA	2,270.1	(7.73)	4,982.9	(81.1)
androst-5-ene-diol	79.55	(0.27)	230.28	(3.75)
androstenedione	54.65	(0.18)	139.3	(2.27)
11β-hydroxyandrostenedione	0		0	
testosterone	357.6	(1.22)	142.5	(2.32)
Phenolic steroid fraction	23.55	(0.08)	67.22	(1.09)
oestrone	0		0.61	(0.01)
oestradiol-17β	0.29	(<0.01)	1.01	(0.02)
oestriol	0		0	
Conjugated steroid fraction	22,353	(78.8)	46.33	(0.75)
DHA sulphate	25,750	(87.6)	0	
androst-5-ene-diol sulphate	48.93*	(0.17)	0	
testosterone sulphate	2.22*	(0.007)	0	

* uncorrected for losses.

Table 16. Evidence for the identity of the steroids isolated from the incubation.

Sertoli cell tumour (2).

Material investigated and chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	Specific activities (dpm/mμmole) ^{3}H	^{14}C
Neutral steroids.				
1. DHA				
-	DHA	A	2,148.5	4,904.2
acetylation	DHA acetate	B	2,165.5	4,609.0
reduction	androst-5-ene-diol	A	2,223.9	4,837.6
2. androst-5-ene-diol				
-	androst-5-ene-diol	C	74.9	215.1
acetylation	androst-5-ene-diol diacetate	E	78.8	230.0
3. androstenedione				
-	androstenedione	A	53.0	134.0
reduction	testosterone	A	52.4	126.3
acetylation	testosterone acetate	A	50.9	138.2
4. 11β-OH androstenedione				
oxidation	adrenosterone	R	0	0
reduction	11-oxo-testosterone	S	0	0
acetylation	11-oxo-testosterone acetate	T	0	0
5. testosterone				
-	testosterone	A	352.3	131.6
acetylation	testosterone acetate	A	341.4	142.6
oxidation	androstenedione	A	336.1	136.3

Table 16 (Continued). Sertoli cell tumour (2).

Material investigated and chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	Specific activities (dpm/mμmole) ^{3}H	^{14}C
Phenolic steroids.				
1. oestrone				
-	oestrone	H	0	0.58
reduction	oestradiol-17β	H	0	0.51
2. oestradiol-17β				
-	oestradiol-17β	H	0.25	0.95
acetylation	oestradiol-17β-diacetate	M	0.17	0.66
saponification	oestradiol-17β	H	0.37	1.15
3. oestriol				
-	oestriol	L		
methylation	oestriol-3-methyl ether	L	0	0
Conjugated steroids.				
1. DHA sulphate				
-	DHA sulphate	J	31,843	0
solvolysis	DHA	A	*	*
acetylation	DHA acetate	B	33,555	0
reduction	androst-5-ene-diol	A	35,026	0
2. androst-5-ene-diol sulphate				
-	androst-5-ene-diol sulphate		*	*
solvolysis	androst-5-ene-diol	A	68.51	0
acetylation	androst-5-ene-diol diacetate	E	59.37	0
3. testosterone sulphate			*	*
-	testosterone sulphate	J	2.77	0
solvolysis	testosterone	A	2.89	0
acetylation	testosterone acetate	A	2.99	0
oxidation	androstenedione	A		

Table 17

Sertoli cell tumour (2)/Normal testicular tissue (1).

To compare the relative amounts of radioactivity isolated with the various steroids investigated in the incubations of tissues from a Sertoli cell tumour and from a normal dog testis. The figures are percentages of the initial radioactivity.

	From 7α-^{3}H DHA sulphate		From 4-^{14}C DHA	
	Tumour	Normal	Tumour	Normal
DHA	7.73	5.59	81.1	39.2
androst-5-ene-diol	0.27	1.30	3.75	17.0
androstenedione	0.18	0.18	2.27	2.77
11β-hydroxyandrostenedione	0	※	0	※
testosterone	1.22	1.07	2.32	21.8
oestrone	0	※	0.01	※
oestradiol-17β	<0.01	※	0.02	※
oestriol	0	※	0	※
DHA sulphate	87.6	93.0	0	0.10
androst-5-ene-diol sulphate	<0.17	※	0	※
testosterone sulphate	>0.007	0	0	0.03

※ not investigated.

the tumour tissue shows far less ability to metabolize DHA (19%) when compared with normal testicular tissue (61%). The conversion of this steroid to the other C_{19}-steroid metabolites investigated is, therefore, considerably less, with the notable exception of androstenedione which shows a formation of 2.27% for the tumour and 2.77% for the normal tissue. This may be contrasted with the results achieved with the Sertoli cell tumour (1) (see Section IV, L iii) in which DHA was converted to a large degree (38%) to androstenedione. In the present experiment the conclusion to be drawn is that of relative dysfunction of the DHA-3β-hydroxydehydrogenase--isomerase. It is noteworthy that what is considered to be the most important androgenic hormone, testosterone, was formed in the tumour at only about one--tenth the amount of that in the normal tissue and invites speculation on the emasculinization that this defect may provoke irrespective of a positive feminization by any oestrogens produced. That the production of oestrogens is also shown to be of a very low level complies with the clinical findings that the dog was not feminized. The alopecia,

however, is not so easily explained in that the evidence already cited (see Sections III and IV, L iv c) indicates oestrogens as the cause of the hair-loss in affected animals. The formation of oestrone and oestradiol-17β from DHA by the normal dog testis described in Section IV, L iv is, admittedly, slightly less than the production found with this tumour but the differences are such, that, within experimental error, they cannot logically be contrasted. A further factor which could be invoked for an explanation is the relative size of the two organs. The tumour weighed 70 g. compared with 10 - 20 g. for the normal testis. The total production of oestrogenic hormones by the tumour may, therefore, exceed that from the normal testis by a factor of 5. Using this argument we must also conclude that testosterone production by the tumour is approximately one-half that of the normal dog testis. The author has seen several dogs suffering widespread alopecia after bilateral orchidectomy and pondered the protection that normal testicular secretions provide against this condition.

No detectable ^{14}C counts were found in the DHA sulphate investigated, indicating inability of this tissue to sulphate DHA. This factor, along with the lowly production of oestrogens by this tissue, lends support to the hypothesis forwarded after the human tumour incubation (see Section IV, L i h) that sulphation of some neutral steroids may be an important factor in the production of oestrogens by this type of feminizing tumour. The conversion figures of DHA to DHA sulphate in the two investigations with normal canine testicular tissue (see Sections IV, L ii and IV, L iv) are 0.10 and 0.13% respectively.

The abilities of both normal and neoplastic tissues to metabolize DHA sulphate are of comparable order. It is of particular interest that this conjugate should have been converted to testosterone sulphate directly without the intermediation of free DHA, androst-5-ene-diol, androstenedione or testosterone, all of which steroids were carbon--labelled. Text fig. 8 indicates the possible pathways by which this transformation could have occurred. Evidence is also provided for the

formation of a tritiated androst-5-ene-diol sulphate by this tissue but whether this was the 3β-mono, 17β-mono or the 3β,17β-disulphate cannot be deduced from the data.

Lebeau & Baulieu (1963) demonstrated the conversion of testosterone to testosterone sulphate by an adrenal tumour, while Dixon, Philips & Kase (1965a) isolated testosterone sulphate after incubating progesterone with a virilizing adrenal tumour but found no free testosterone. Dixon et al. (1965) considered the possibility of a sulphated intermediate being a precursor of testosterone sulphate rather than the direct sulphation of testosterone in the light of the findings of Wallace & Silberman (1964) of androst-5-ene-diol disulphate from an ovarian incubation. They concluded that this latter compound was not an intermediate and that direct sulphation of testosterone had occurred.

Lipsett, Sarfaty, Bardin & Fishman (1966) indicated by their in vivo studies of a man with a metastatic interstitial cell carcinoma of the testis, that testosterone sulphate was formed from

infused 7α-^{3}H DHA sulphate. They also suggested that the plasma DHA sulphate of their patient was the sole precursor of the urinary androst-5-ene--diol sulphate and 16α-hydroxy DHA sulphate, in agreement with the findings of Roberts et al. (1964b).

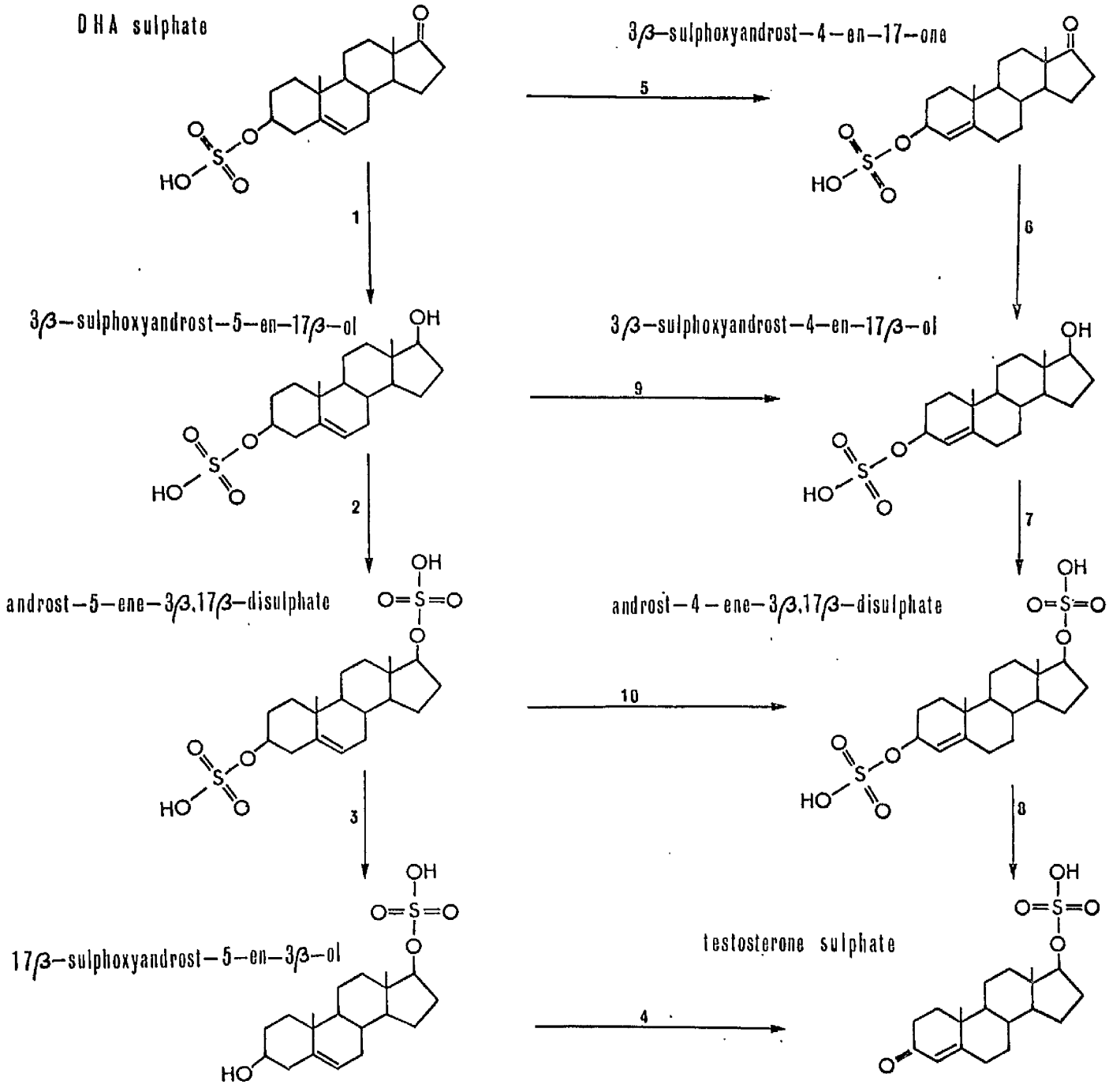
The most likely pathway for the transformation of DHA sulphate to testosterone sulphate, in the light of the results obtained, would seem to be by reduction of the 17-oxo-group (reaction 1, Text fig. 8) to form androst-5-ene-diol-3-sulphate and the further conjugation of this compound (reaction 2) to give androst-5-ene-diol disulphate. Selective hydrolysis (reaction 3) achieves androst-5-ene-diol-17-sulphate which is converted by a 3β-hydroxysteroid-dehydrogenase-isomerase (reaction 4) to testosterone sulphate.

At any stage the conversion of these Δ^5 3β-hydroxy Δ^4 steroids[illegible]sponding 3β-sulphoxy or 3β-hydroxy Δ^4 steroids (reactions 5, 9, 10) must be considered a possibility as none of these compounds will mix with the pool of known ^{14}C--labelled steroids that were isolated.

A sulphated form of androst-4-ene-diol was

Text–figure 8.

Possible pathways to the formation of testosterone sulphate from DHA sulphate.



not demonstrated in the experiment with normal testicular tissue (see Section IV, L ii).

Baulieu & Corpechot (1965) described the direct conversion in vivo of DHA sulphate to androst-5-ene-diol disulphate without cleavage of the sulphate group. Payne & Mason (1965b) showed that soluble extracts of rat testis could convert DHA sulphate to androst-5-ene-diol-3--sulphate and that this conversion was four times more efficient than the similar reaction involving the free steroids.

An alternative explanation for the transformation of DHA sulphate to testosterone sulphate is via the more orthodox pathway of DHA sulphate ⟶ DHA ⟶ androstenedione (and/or androst--5-ene-diol) ⟶ testosterone ⟶ testosterone sulphate but, due to compartmentalization within the testicular cells, these intermediates are not allowed to mix with those metabolites of unconjugated 4-^{14}C DHA. This sequence is considered less probable than the scheme via sulphated steroids.

Testosterone sulphate was isolated from the incubation with the normal testicular tissue (see

Section IV, L ii d) but possessed only the carbon--label and is considered to be a direct product of the sulphation of testosterone; the tritium content of which was of such low magnitude that the experimental procedure could not detect its presence in the conjugate. This finding complies with that of Dixon et al. (1965) with normal human testicular tissue.

The relative ratios of ^{14}C to ^{3}H present in the androst-5-ene-diol, androstenedione and testosterone isolated from the two incubations described allow for interesting speculation as corollary to the foregoing discussion. These ratios from the normal testis experiment are, respectively, 13:1, 15:1 and 20:1 showing, as mentioned earlier, a much better utilization of DHA than of its sulphate ester. The tumour experiment, however, provides respective ratios of 14:1, 13:1 and 2:1 indicating not only almost identical values for androst-5-ene-diol and androstenedione as those from normal testis, but a reduction in the ratio of ^{14}C to ^{3}H for testosterone by one-tenth. This is due to the greater tritium content (1.22%)

of the testosterone as compared with 0.27% (androst-5-ene-diol) and 0.18% (androstenedione) rather than a lower incorporation of ^{14}C into its molecule. It is suggested that this tritium could be derived from hydrolysis of the testosterone sulphate arising directly from 7α-^{3}H DHA sulphate as described earlier. If this were to be the case it would provide evidence, for the first time, for the presence of a sulphatase enzyme capable of hydrolysing a 17β-sulphate. Baulieu, Corpechot, Dray, Emiliozzi, Lebeau, Mauvais-Jarvis & Robel (1965) and Jarrige (1962) have asserted that all experimental evidence indicates, as yet, that the ester-sulphates of a 17β- alcohol grouping are not hydrolysed.

The 11β-hydroxyandrostenedione isolated during this investigation was found not to be radio-actively labelled indicating its non-production by this tissue. Androstenedione-11β-hydroxylase has been found in abnormal testicular tissue from man (Savard et al., 1960), although its steroid specificity was indicated by its failure to act on progesterone. Lipsett et al. (1966) indicated a

similar steroid specificity of this enzyme after isolating 11β-hydroxyandrosterone from a patient's urine but found no 11-oxopregnanetriol. The presence of such an enzyme in the tissue described here might then have been missed were it to be active on a steroid other than androstenedione and which, after hydroxylation, was not further metabolized to the particular derivative investigated here.

(vi) Sertoli cell tumour (3).

An investigation of the metabolism, in vitro, of 4-^{14}C DHA and 7α-^{3}H DHA sulphate by a Sertoli cell tumour of the testis from a dog with signs of feminization but no alopecia.

(a) Clinical data.

The patient, a 4 yr. old Shetland collie was presented for veterinary advice because of the attention paid to it by other male dogs. The owner reported that up to half-a-dozen dogs would attend the house as if his dog were a bitch on heat. Examination revealed a young well-kept animal with a good thick coat and a large swelling in the right inguinal region. The prepuce was somewhat turgid and did not hold the usual horizontal position of the normal organ. There was some slight enlargement of the teats, particularly those more posteriorly placed. No atrophy of the penis was found. The left testis was in the scrotum, both of which organs were atrophied.

The dog was submitted for surgery and, under general anaesthesia (intravenous thiopentone Na/ /cyclopropane/O_2), the right testis was removed from the inguinal region. The left testis was excised at the same time. The tumour, weighing 31.0 g. and measuring 5 x 4 cms. was hard and fibrous and showed a creamy-white lobulated appearance on section. The left testis was atrophic and weighed 2.0 g.

(b) Histological description.

Basically, this tumour was of a tubular structure (Plate 4) though the tissue was particularly solid in appearance. In the greater part of the section, the tumour was made up of very small tubules separated by a finely distributed fibrous tissue stroma. The architecture, however, varied considerably in different areas. In places the cells were arranged in columns and islands, and even when tubule formation was more obvious, a central lumen was often absent. Usually the cells were several layers deep. A number of very

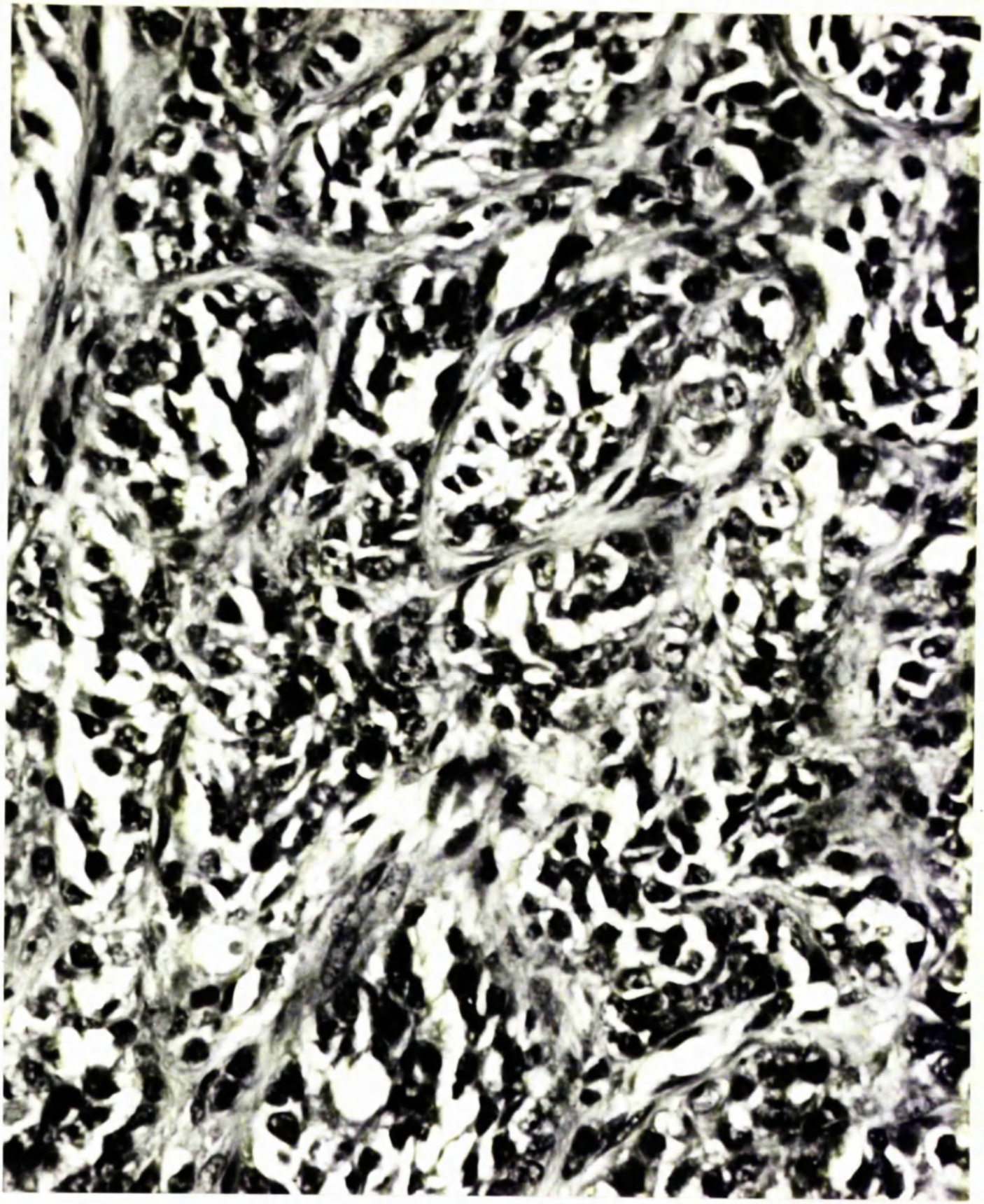


PLATE 4. Sertoli cell tumour (3).

(x 500)

large tubules were present and some of these were cystic, while there was no cavity in others. Abundant intra-cytoplasmic Sudanophilic lipid was demonstrated in the tumour cells. The mitotic rate was moderately high. This was a Sertoli cell tumour.

(c) Preparation of the tissue and conditions of incubation.

The tumour was transferred to the laboratory in crushed ice and prepared for incubation (see Section IV, B iii). The chopped tissue (2 g.) was suspended in 24 ml. Krebs Ringer bicarbonate glucose solution and incubated, as was the last tumour, with 72.7 mμmoles each of 7α-^{3}H DHA sulphate (206.3 μC/μmole) and 4-^{14}C DHA (27.5 μC/ /μmole) for 2.5 hr. The only difference in the technique adopted for this tissue from that of the last tumour and the 2.5 hr. incubation of the time--controlled experiment (see Section IV, L ii a) was the temperature of incubation. The tumour reported here had been ectopically placed and was therefore incubated at body temperature, 38.6^{o}.

The reaction was stopped by the addition of 10 ml. acetone and refrigerating at -15°.

The following steroids were added as cold carriers, 300 µg. in each case, DHA, androst-5--ene-diol, androstenedione, testosterone, DHA sulphate, testosterone sulphate, oestrone, oestradiol-17β, oestradiol-17α and oestriol.

Extraction Procedure II (see Section IV, D ii) was employed to extract and fractionate the steroids from the incubation medium.

(d) Investigation of the various steroid fractions.

The steroids from the neutral and conjugated steroid fractions were isolated and measured as described for the normal canine testicular tissue (1) (see Section IV, L ii b & c). The phenolic fraction was dealt with as described for the Sertoli cell tumour (1) (see Section IV, L iii e).

(e) Results.

The recovery of radioactivity in the various

Table 18

Recovery of radioactivity in the various fractions and the total radioactivity (dmp x 10^{-3}) associated with the steroids isolated from the incubation of Sertoli cell tumour (3).

Percentages of the initial radioactivity are shown in parentheses.

	From 7α-^{3}H DHA sulphate		From 4-^{14}C DHA	
Neutral steroid fraction	3,117.17	(10.5)	4,730.2	(86.8)
DHA	2,319.1	(7.8)	3,228.6	(59.3)
androst-5-ene-diol	7.241	(0.02)	15.73	(0.29)
androstenedione	505.5	(1.70)	1,186.4	(21.8)
testosterone	47.92	(0.16)	104.58	(1.92)
Phenolic steroid fraction	31.25	(0.11)	29.73	(0.55)
oestrone	0		0.8	(0.01)
oestradiol-17β	0		0.63	(0.01)
oestradiol-17α	0		0	
oestriol	0		0	
Conjugated steroid fraction	20,002	(67.3)	91.81	(1.69)
DHA sulphate	27,179	(91.5)	5.92	(0.11)
testosterone sulphate	1.95	(0.007)	0	

Table 19. Evidence for the identity of the steroids isolated from the incubation of Sertoli cell tumour (3).

Material investigated and chemical reaction	Chromatographic mobility identical with that of:-	Solvent system	Specific activities (dpm/mμmole) ^{3}H	^{14}C
Neutral steroids.				
1. DHA				
-	DHA	A	2,221.9	3,024.1
acetylation	DHA acetate	B	2,278.9	3,181.2
reduction	androst-5-ene-diol	A	2,178.1	3,093.3
2. androst-5-ene-diol				
-	androst-5-ene-diol	C	7.1	15.2
acetylation	androst-5-ene-diol diacetate	E	7.0	15.2
3. androstenedione				
-	androstenedione	A	494.1	1,167.6
reduction	testosterone	A	466.0	1,159.1
acetylation	testosterone acetate	A	485.5	1,066.3
4. testosterone				
-	testosterone	A	46.5	103.5
acetylation	testosterone acetate	A	47.9	98.5
oxidation	androstenedione	A	43.5	99.1
Phenolic steroids.				
1. oestrone				
acetylation	oestrone acetate	M	0	0.73
methylation	oestrone-3-methyl ether	M	0	0.72
2. oestradiol-17β				
-	oestradiol-17β	H	0	0.50
acetylation	oestradiol-17β-diacetate	M	0	0.73
methylation	oestradiol-17β-3-methyl ether	L	0	0.49
3. oestradiol-17α				
acetylation and saponification	oestradiol-17α	H	0	0
4. oestriol				
acetylation and saponification	oestriol	L	0	0
Conjugated steroids.				
1. DHA sulphate				
-	DHA sulphate	J	33,652	*
solvolysis	DHA	A	36,968	7.82
acetylation	DHA acetate	B	36,415	8.64
reduction	androst-5-ene-diol	A	34,298	6.63
2. testosterone sulphate				
-	testosterone sulphate	J		
solvolysis	testosterone	A	2.06	0
acetylation	testosterone acetate	A	3.44	0
oxidation	androstenedione	A	2.12	0

* unsatisfactory result.

Table 20

To compare the metabolism of 4-^{14}C DHA by normal and neoplastic testicular tissue.

	Normal testicular tissues		Sertoli cell tumours		
	1.	2.	1.	2.	3.
DHA (unchanged)	39.2	35.2	42.2	81.1	59.3
androst-5-ene-diol	17.0	19.2	1.34	3.75	0.29
androstenedione	2.77	5.62	38.2	2.27	21.8
testosterone	21.8	26.2	6.08	2.32	1.92
oestrone	⁎	<0.01	0.30	0.01	0.01
oestradiol-17β	⁎	<0.01	0.17	0.02	0.01
DHA sulphate	0.10	0.13	0.18	0	0.12
testosterone sulphate	0.03	⁎	⁎	0	0

⁎ not investigated.

steroid fractions and the total radioactivity associated with steroids investigated are shown in Table 18. The evidence for the identification of the steroids isolated is given in Table 19 and two comparative tables (Tables 20 & 21) have been drawn up to compare and contrast the results from this experiment with those from the investigations of the foregoing Sertoli cell tumours and the normal testes.

(f) Discussion.

It may be seen from Table 20 that the formation of oestrogens, in vitro, by this feminizing tumour is of the same order as that for the non-feminizing tumour of the last section, and somewhat greater than that provided by the normal testicular tissue (see Section IV, L iv b). It would be surprising if such a low level were responsible for the clinical signs observed even with due consideration of the total mass (31 g.) of the tumour when it is realised that the tumour associated with alopecia (see Section IV, L v), producing similar quantities of oestrogens, weighed more than twice as much (70 g.)

and yet produced no feminizing signs.

It is of particular interest that the transformation of 4-^{14}C DHA to testosterone and androstenedione should again favour the latter steroid as with the Sertoli cell tumour (1) investigated (see Section IV, L iii). The conversion of DHA to androstenedione (22%) in the present investigation greatly exceeds that achieved by the two incubations with normal testicular tissue (Table 20), in which reduction to testosterone (22 and 26% respectively) would appear to have been much more efficient. A decrease in amount or efficiency of androstenedione-17-oxo-hydrogenase is indicated in this tumour tissue.

Considering the lowly production of oestrogens by this tumour and the efficient conversion, both in this tissue and in the previous feminizing tumour (see Section IV, L iii), of DHA to androstenedione, as compared with the normal tissues and the non-feminizing tumour, it would suggest that the androstenedione, instead of being converted to the potent androgen, testosterone,

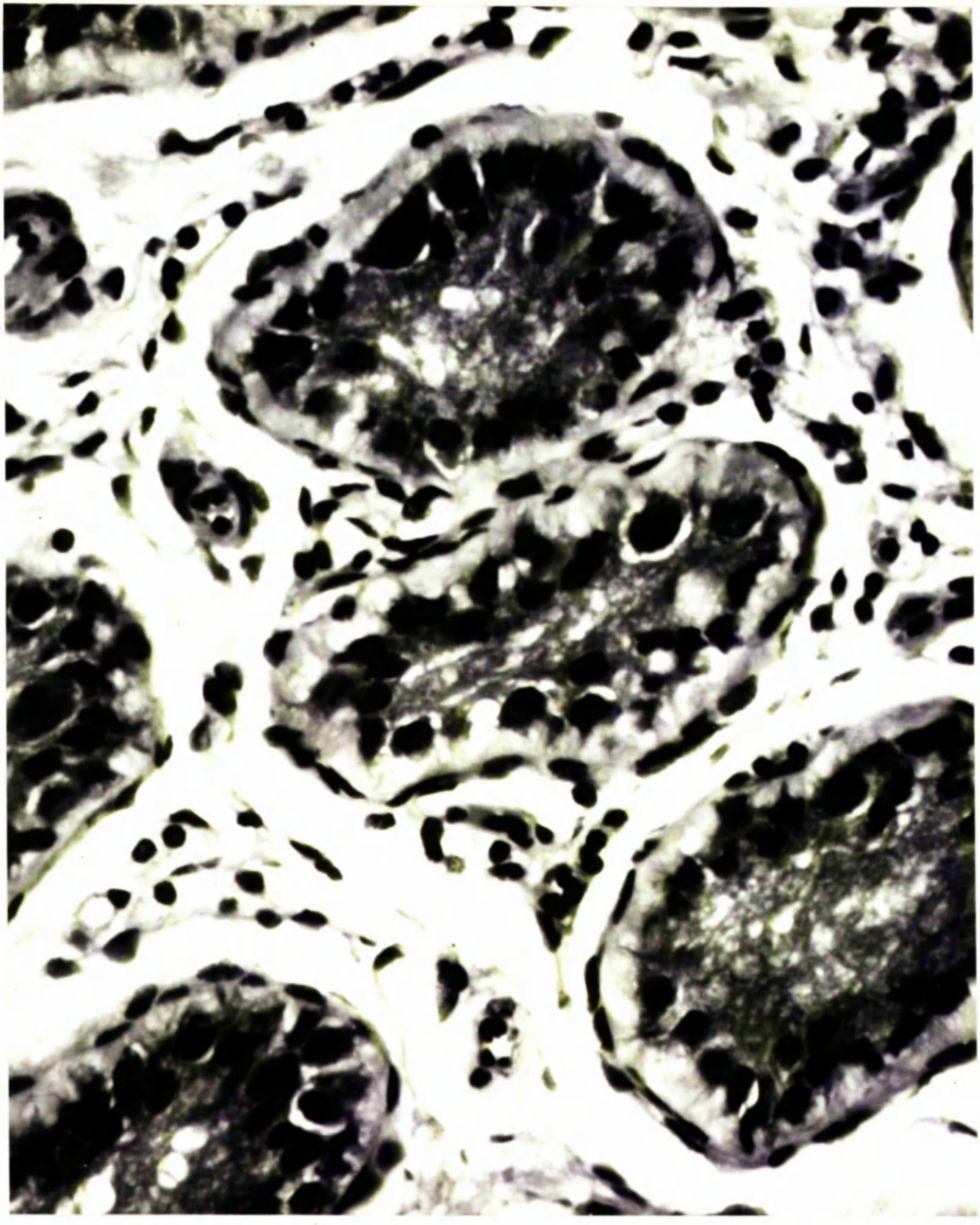


PLATE 5. Atrophic testis contralateral to Sertoli cell tumour (3).

(x 800)

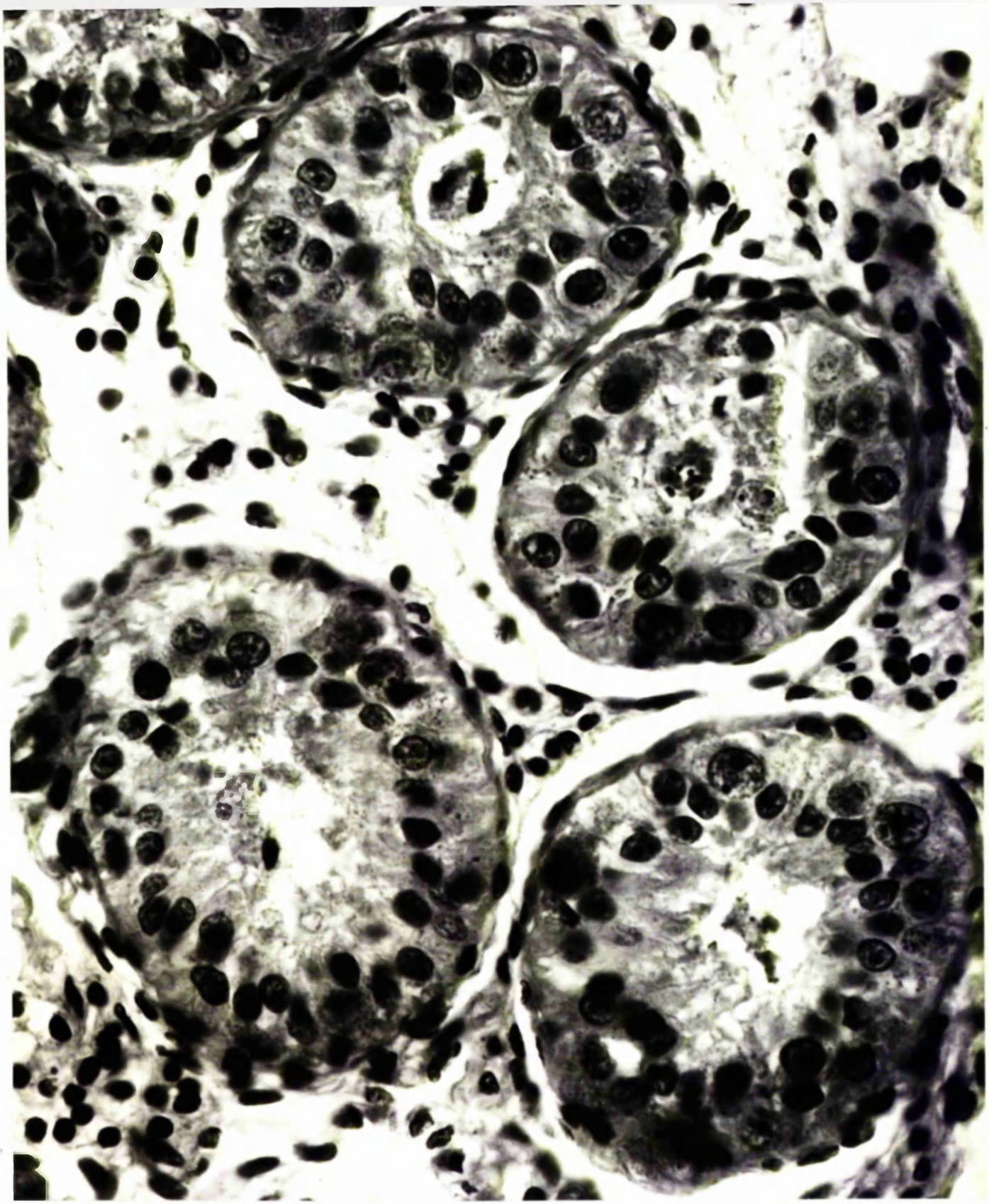


PLATE 6. Atrophic testis from dog on oestrogen therapy.

(x 800)

in the testis, is being secreted in the blood stream and converted to oestrogens elsewhere in the body. West, Damast, Sarro & Pearson (1956) and Braun--Cantilo, La Roche, Novitsky & Lawrence (1961), have shown the production of oestrogens in vivo after the injection of androgens into adrenalectomized and oöphorectomized subjects. Recently Kase & Cohn (1967) described a patient with absent pituitary-ovarian function in whom it was concluded that DHA sulphate, secreted by the adrenal gland, was being converted to oestradiol-17β peripherally.

Histological examination (Plate 5) of the contralateral "normal" testis from this dog provides further evidence that this animal was suffering a hyperoestrogenism. The seminiferous tubules of this organ are seen to contain Sertoli cells and spermatogonia only, with no evidence of spermatogenesis. The lumina of the tubules are occupied by amorphous basophilic material, interspersed among which are scattered pyknotic nuclei. There is marked thickening of the tubular basement membrane and lamina propria. Few interstitial cells are

seen. The appearance is that of an atrophic testis and is considered to be consistent with the effects of oestrogen suppression. Similar findings have been reported (De La Balze, Gurtman, Janches, Arrillaga, Alvarez & Segal, 1962) for the human testis after the administation of diethylstilboestrol to men with prostatic cancer.

Plate 6 shows the histological appearance of a testis from a dog that had received 1 mg. oestradiol benzoate daily for a period of 3 mth. The resulting atrophy demonstrated appears, in fact, less severe than in the case of the non--neoplastic organ from the dog described in this section indicating the suppressive activity that was induced by the Sertoli cell tumour, either by direct or indirect means.

This tumour tissue showed a much higher metabolic activity with regard to DHA (40%) than did the previous tumour (20%) and approached more closely that of the normal tissues (60 and 65% respectively). The Sertoli cell tumour (1) also had a high metabolic activity with a 58% trans-

formation of DHA.

In contradistinction to the non-feminizing tumour, DHA sulphate was formed from DHA although ^{14}C-labelled testosterone sulphate was not isolated. Evidence is provided, however, for the formation of tritiated testosterone sulphate indicating a similar metabolic pathway from 7α-^{3}H DHA sulphate to that described in the last section (see Section IV, L v e, and Text fig. 8).

The metabolism by this tumour, of 7α-^{3}H DHA sulphate shows interesting similarities and differences to that of the previous tumours. The amount of this steroid that was isolated unchanged after the 2.5 hr. incubation period was high (approx. 90%) in all three investigations where it was employed as a precursor. The two tumours provided almost exactly the same conversion to DHA (7.7 and 7.8% respectively, Table 21) which was slightly better than the conversion provided by the normal tissue (5.6%). The metabolism from this stage on, however, differed considerably in all three tissues. Androstenedione was again

Table 21

To compare the metabolism of 7α-^{3}H DHA sulphate by normal and neoplastic testicular tissue.

	Normal testicular tissue (1)	Sertoli cell tumours (2)	(3)
DHA sulphate (unchanged)	93.0	87.6	91.5
DHA	5.59	7.73	7.81
androst-5-ene-diol	1.30	0.27	0.02
androstenedione	0.18	0.18	1.70
testosterone	1.07	1.22	0.16
testosterone sulphate	0	>0.007	0.007

the predominant product from the feminizing tissue, as with the previous feminizing Sertoli cell tumour (see Section IV, L iii), exceeding the equivalent formation of this steroid by the tumour associated only with alopecia by a factor of 10 (Tumour 2, Table 20). The formation of testosterone was again very low. The results also indicate that once hydrolysis of the 7α-^{3}H DHA sulphate has occurred the metabolism of the resulting DHA continues as for the unconjugated substrate, 4-^{14}C DHA.

The essential differences between the tumours causing feminization and the one unassociated with such signs appears to be a greater steroidogenic activity, a DHA-sulphating enzyme system and a higher androstenedione:testosterone production in the former (Tables 20 & 21).

It is not easy to explain the cause of the feminization that was demonstrated in this final case nor the reason for apparent oestrogenic--suppression of the contralateral testis although hypotheses have been presented. Greater difficulty is found in understanding the reason for the atrophy observed in the "normal testis" from the Cairn

terrier with the Sertoli cell tumour (2) as this animal had no signs of feminization.

Why some Sertoli cell tumours cause feminization, others alopecia and 83% (Dow, 1962; Table 2) or 71% (Cotchin, 1960; Table 2) are associated with no discernible endocrine changes at all, may be decided upon by further investigations.

V. Summary and concluding remarks.

The metabolic activity particular to certain testicular cell-types and the changes thereof that become apparent with the advent of neoplasia, coincident with the species-different systemic effects manifested, has prompted the work described in this Thesis. The canine Sertoli cell tumour and the human interstitial cell tumour are of special interest in this respect with regard to their providing neoplastic tissue of one cell-type and being commonly associated with feminization in the host animal.

The metabolism *in vitro* of certain radioactive isotopically-labelled steroids by one human and three canine testicular tumours has been investigated. It is considered that the results obtained provide interesting information concerning the steroidogenic activity of these tumours and of the particular neoplastic cell-type. Tissue from two normal canine testes has been incubated in identical manner for comparative purposes. Normal human testes are not easily obtained.

The transformation of 4-^{14}C DHA to oestradiol--17β and of 7α-^{3}H pregnenolone to oestriol has been demonstrated in tissue from an interstitial cell tumour from a man with gynaecomastia. The production of oestriol by a pathway not involving DHA or oestradiol-17β has prompted the hypothesis that 16α-hydroxylated neutral steroids, either free or conjugated, may be of some importance in the synthesis of this steroid by the neoplastic interstitial cells.

It was also shown that this tumour was capable of converting certain neutral steroids to their sulphate esters. The role of steroid sulphates has assumed greater importance during the past decade than hitherto anticipated with regard to their further participation in biosynthetic sequences to steroid hormone production. It is postulated that their formation in this feminizing tissue may play some part in the oestrogen synthesis demonstrated.

New methods are also described for the isolation and separation of certain neutral steroid sulphates using thin-layer chromatographic techniques.

Tissue from a canine Sertoli cell tumour has been shown, for the first time, to synthesize oestrone

and oestradiol-17β from 4-^{14}C DHA. The comparative studies made of the metabolism of this tumour type and of tissue from normal dog testis has indicated relative enzyme inactivity in the tumour, and, in particular, has provided evidence for steroid--specific 17α-hydroxylase, 16α-hydroxylase and 3β-hydroxysteroid dehydrogenase-isomerase enzyme systems in canine testicular tissue.

The Sertoli cell has, in the past, been discounted, on the whole, ~~as~~ of being capable of steroid metabolism and synthesis, yet the results of these investigations indicate the presence in this cell of several enzyme systems capable of performing these functions. Pregnenolone was shown to be converted to 17α-hydroxypregnenolone, progesterone, DHA, androst-5-ene-diol and testosterone. The sulphation of neutral steroids was also demonstrated in this tissue, and a suggested pathway to the synthesis of testosterone sulphate from DHA sulphate is described.

Recently Woods & Domm (1966), using a fluorescent steroid-specific antibody technique, have indicated that the Sertoli cells of the rat and fowl testis can synthesize androgens.

The ability of normal and neoplastic canine testicular tissue to transform DHA sulphate to testosterone, androst-5-ene-diol and androstenedione *in vitro* has also been demonstrated.

Differences are noted between the ability of a canine Sertoli cell tumour described herein and that reported for two similar tumours of human origin (Stempfel, quoted by Lacy, 1962; and Griffiths *et al.*, 1963) to synthesize 17α-hydroxyprogesterone and oestrogens, and the question is raised as to the significance these findings may have in relation to the relative tendencies to malignancy shown by this tumour in the two species.

It is considered that differences observed in the metabolism of DHA by the two feminizing canine Sertoli cell tumours as opposed to the tumour unassociated with such signs may not only be of significance with regard to clinical signs observed but also with respect to the degree of malignancy of the tumours. Moulton (1961) concluded that the histological appearance of this type of tumour shows no constant correlation with malignancy thus providing difficulty in classifying benign and

malignant growths. This author does indicate, however, that malignancy tends to be associated with special histological features such as the pleomorphic appearance of the Sertoli cells, the possession, by them, of larger, more hyperchromatic nuclei and the tendency of the neoplastic cells to infiltrate rather than to remain in the tubules. These criteria are observable in Plates 2 and 4 from the two feminizing tumours in contrast to the benign appearance of the tumour associated only with alopecia (Plate 3). It is of interest that the more anaplastic the cells are the greater their steroidogenic activity seems to be. The biochemical results, such as the androstenedione: :testosterone production, obtained from an incubation of these tissues with a substrate such as $4\text{-}^{14}C$ DHA could be of some ancillary aid in the differential diagnosis of benign from malignant growths.

VI. Appendices.

Appendix 1.

Preparation of Krebs-Ringer bicarbonate-glucose medium.

The method of preparation of this medium is based on that described by Cohen (1957).

	Volume (ml.)	Concentration of solutions M.	g./100 ml.	Final conc. M.
NaCl.	10	1.16	6.78	0.116
KCl.	10	0.465	3.47	0.0465
$NaHCO_3$ *	10	0.244	2.05	0.0244
glucose	10	-	1.0	-
KH_2PO_4	1	0.116	1.573	0.00116
$MgSO_4.7H_2O$	1	0.116	2.86	0.00116
H_2O	57	-	-	-
$CaCl_2.6H_2O$	1	0.242	5.3	0.00242

The final volume = 100 ml.

*previously gassed for 1 hr. with CO_2.

The final mixture is gassed for 10 min. with 5% CO_2 in oxygen, after which the pH is generally found to be 7.4.

Appendix 2.

Sources of chemicals.

Common reagents	-	British Drug Houses, Ltd., Poole, Dorset (ANALar grade unless otherwise stated).
Nucleotides	-	Boehringer & Soehne GmbH., Mannheim, W. Germany.
Glucose-6-phosphate--dehydrogenase	-	Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Radioactive steroids.

Radioactive steroids were purchased from the Radiochemical Centre, Amersham, Bucks., and were stored at -15^{o} at an approximate concentration of 1 μC/ml. (^{14}C-steroids) or 10 μC/ml. (^{3}H-steroids) in a mixture of methanol:benzene (1:9 v/v). The purity of these compounds as claimed by the makers was checked by thin-layer chromatography and scanning with a Packard Radiochromatogram Scanner, Model 7200.

Non-labelled steroids.

These steroids were obtained from Koch-Light Laboratories, Ltd., Colnbrook, Bucks., Steraloids Ltd., Croydon, Surrey, Ikapharm, Ramat-Gan, Israel and Mann Research Laboratories, Inc., New York, U.S.A. The steroid sulphates were kindly donated by Dr. G.F. Woods and Dr. C.L. Hewett, Organon Laboratories Ltd.

Solvents.

Ethanol and methanol (Burroughs, A.R. grade) and diethyl ether, acetone, petroleum ether and toluene were used without further purification. It was found necessary to purify the benzene by shaking with conc. H_2SO_4. Traces of acid were then removed by washing with water, and after drying over anhydrous $CaCl_2$ the benzene was distilled twice. All other solvents used were washed with water and redistilled before use. These procedures appeared to minimize interference with spectrophotometric analysis.

Appendix 3.

To achieve a reasonable extraction of neutral steroid sulphates and phenolic steroids from incubation media.

In this investigation DHA sulphate and oestrone were taken as representatives of, respectively, neutral steroid sulphates and phenolic steroids.

Unlabelled DHA sulphate and oestrone, 300 μg. in each case, were added to a mixture of 0.5 μC. each of 7α-^{3}H DHA sulphate and 4-^{14}C oestrone in a flask containing 2 g. minced guinea pig testis in 24 ml. water and 10 ml. acetone. This was considered to simulate an incubation medium prior to extraction.

The steroids were extracted using Extraction Procedure II (see Section IV, D ii). The procedure for separating neutral and phenolic steroids (20 ml. N.NaOH/6 x 20 ml. toluene) was performed and the alkaline residue re-extracted with 3 x 20 ml. ether after adjusting to pH 8.

The steroid sulphate was extracted from the aqueous phase by the method of Edwards et al. (1953) as described earlier (see Section IV, D ii).

The procedure adopted for assessing the amounts of radioactivity in the various fractions was as follows:-

1. Alkali fraction.
   (a) 1/10 volume dried in a vial and taken up in 10 ml. scintillator solution.
   (b) 1/10 volume dried and taken up in 1 ml. methanol + 9 ml. scintillator solution.

2. Aqueous fraction.
   (a) As for 1a.
   (b) As for 1b.

3. Toluene fraction.
   (a) As for 1a.
   (b) As for 1b.

The radioactivity was measured and assessed for both the quenched and unquenched aliquots as described in Section IV, J.

Results.

Total initial radioactivity:-

7α-^{3}H DHA sulphate - 1,959,100 dpm.

4-^{14}C oestrone - 1,386,200 dpm.

The total amounts of radioactivity, as assessed by the two techniques of counting, in each extract. Percentages of initial radioactivity are in parenthesis.

Fraction	^{3}H	^{14}C
1a	0	423,600 (30.6)
1b	0	421,400 (30.4)
2a	1,444,200 (73.7)	3,100 (0.2)
2b	1,485,100 (75.8)	5,000 (0.4)
3a	30,000 (1.5)	704,700 (50.8)
3b	13,800 (0.7)	707,700 (51.1)

It may be seen from the accompanying table that only approximately 1% of the DHA sulphate was located in the toluene or neutral steroid fraction whereas 50% of the oestrone was found there. The recovery of DHA sulphate from the aqueous residue was of the

order of 75% but oestrone counts in the alkaline fraction achieved only 30% of the maximum. The low level of contamination (0.2 - 0.4%) of oestrone in the aqueous fraction was considered satisfactory.

Recovery of the oestrone from the toluene fraction was attempted by back-extracting with N.NaOH. The toluene was evaporated and the dry residue taken up in 20 ml. toluene and extracted with 3 x 20 ml. N.NaOH. These volumes were not bulked but extracted individually with 3 x 20 ml. ether.

Each ether extract of 3 x 20 ml. and the toluene residue were assessed for radioactive content by removing a known aliquot for scintillation counting without the addition of methanol.

The total radioactivity associated with each extract and the percentage of the original radioactive in parenthesis.

Extract	^{14}C (dpm)	Percentage of the ^{14}C originally in the toluene residue
Ether extract (1)	359,300 (25.9)	51.0
Ether extract (2)	88,700 (6.4)	12.6
Ether extract (3)	21,700 (1.6)	3.1
Toluene residue	35,000 (2.5)	5.0

The results shown in the above table indicate that one back-extraction of the toluene fraction with 20 ml. NaOH will remove 50% of the contained oestrone. Subsequent extractions remove 13% and 3% respectively.

These results indicate that the procedure outlined in Extraction Procedure II (Section IV, D ii) provides a good, clean recovery of DHA sulphate in the aqueous phase. Oestrone, on the other hand, tends to be easily extractable from an alkaline medium by toluene. Back-extraction of the toluene with 20 ml. N.NaOH recovers 50% of this oestrone.

To assess the repeatability of the previous investigation.

The "incubation medium" containing labelled and non-labelled DHA sulphate and oestrone was prepared as before and the steroids extracted as described previously.

The toluene extract was reduced to 20 ml. volume and back-extracted with 2 x 20 ml. N.NaOH, keeping each extract separate. The two soda phases were then separately adjusted to pH 8 and the steroids extracted with 3 x 30 ml. ether.

The aqueous residue was dealt with, in respect to DHA sulphate, as described before.

Aliquots of each fraction were taken for counting as before.

Results.

The table shows the total radioactivity in the various fractions. Percentages of the initial radio-activity are given in parentheses.

	^{3}H	^{14}C
Aqueous fraction	2,019,850 (82.3)	13,740 (0.99)
Residual activity in the soda phase after toluene extraction.		351,880 (25.4)
1st back-extraction of the toluene.		524,280 (37.8)
2nd back-extraction of the toluene.		154,730 (11.2)
Residual activity in the toluene.	23,710 (0.97)	39,090 (2.7)

The accompanying table shows again a good recovery (82%) of DHA sulphate but a very poor initial recovery (25%) of oestrone after extracting the NaOH containing this oestrogen with 6 x 20 ml. toluene. Back--extraction of the toluene phase achieved a recovery of 50% giving a total of approximately 75% of the original oestrone.

Appendix 4.

To achieve a reasonable separation of neutral and phenolic steroids after extraction from the incubation media.

In this experiment neutral and phenolic steroids were represented, respectively, by DHA and oestrone.

Unlabelled DHA and oestrone, 300 μg. in each case, were added to 0.5 μC each of 7α-^{3}H DHA and 4-^{14}C oestrone. The mixture was then divided equally into three portions and dried. Toluene (1 ml.) was added to each tube followed by 20 ml. N.NaOH. The steroid mixtures were then subjected to three extraction procedures; tube 1. was shaken with 2 x 20 ml. toluene, tube 2. with 4 x 20 ml. toluene and tube 3. with 6 x 20 ml. toluene.

The toluene fractions were evaporated to dryness and a small aliquot taken from each for counting by liquid scintillation counter. The remainder was taken up in toluene again (20 ml.) and back-extracted with 1 x 20 ml. N.NaOH.

The alkali fractions from the initial and back-extractions were taken, individually, to pH 8

using conc. HCl and steroids extracted with 3 x 20 ml. ether. A known volume from each ether fraction was then taken for counting.

Results.

Total initial radioactivity:-

7α-^{3}H DHA - 1,077,500 dpm.

4-^{14}C oestrone - 1,466,000 dpm.

The total amounts of radioactivity (dpm) and, in parenthesis, the percentages of initial radioactivity in each extract.

	^{3}H		^{14}C	
Before the back-extraction.				
Toluene fraction.				
Tube 1.	1,056,800	(98.1)	454,100	(31.0)
Tube 2.	984,500	(91.4)	725,000	(49.5)
Tube 3.	1,026,900	(95.3)	967,000	(66.0)
Alkaline fraction.				
Tube 1.	0		761,800	(52.0)
Tube 2.	0		643,400	(43.9)
Tube 3.	0		529,800	(36.1)

	^{3}H		^{14}C	
After the back-extraction.				
Toluene fraction.				
Tube 1.	907,000	(84.2)	78,500	(5.35)
Tube 2.	875,600	(81.3)	118,100	(8.06)
Tube 3.	868,700	(80.6)	149,000	(10.2)
Alkaline fraction.				
Tube 1.	136,200	(12.6)	264,600	(18.1)
Tube 2.	143,900	(13.4)	323,100	(22.0)
Tube 3.	216,000	(20.1)	614,300	(41.9)

The accompanying table shows that the DHA is equally well extracted by 2 x 20 ml. toluene as by 4 x 20 ml. or even 6 x 20 ml. In fact the consequence of exceeding that initial procedure is a further withdrawal of oestrone from a 31% to a 66% extraction. The readings obtained with the alkaline fraction confirm these findings indicating that 2 x 20 ml. toluene is optimum.

Back-extracting the toluene with 20 ml. N.NaOH does remove some of the oestrone but also reduces the yield of DHA and carries some of this latter compound back into the otherwise DHA-free alkaline fraction.

It would seem reasonable, therefore, either to extract the soda phase with 2 x 20 ml. toluene and not to back-extract, giving the following values:-

Toluene fraction	-	98% of the DHA 31% of the oestrone.
Phenolic fraction	-	No DHA 52% of the oestrone.

or to back-extract the toluene once or even twice but not to bulk these fractions with the phenolic fraction which is already free of DHA.

The former of these was adopted in order to maintain the high recovery of neutral steroids and use thin-layer chromatography and derivative formation to separate the contaminating phenolic steroids.

Appendix 5.

To assess the effect of an eluate from an ecteola--coated plate pre-run in the solvent system 4M. Urea in 3N.NH_4OH on the quantitation of DHA sulphate using methylene blue.

The final purification of DHA sulphate in these investigations is by chromatography on ecteola--coated plates in solvent system J. (Table 4). In the investigation of the human tumour (see Section IV, L i) the conjugates were eluted with methanol, and in subsequent work by ethyl acetate from saturated NaCl solution (see Section IV, G). To assess any effect that such procedures might have on the quantitation of the steroid conjugates by the methylene blue method (Crepy & Rulleau-Meslin, 1960) the following investigation was carried out.

An ecteola-spread plate was placed in a tank containing the solvent system J. until the solvent front had traversed most of the plate. After drying, the ecteola was divided into areas of approximately 5 sq. cm. being the area that a steroid would have covered after chromatography.

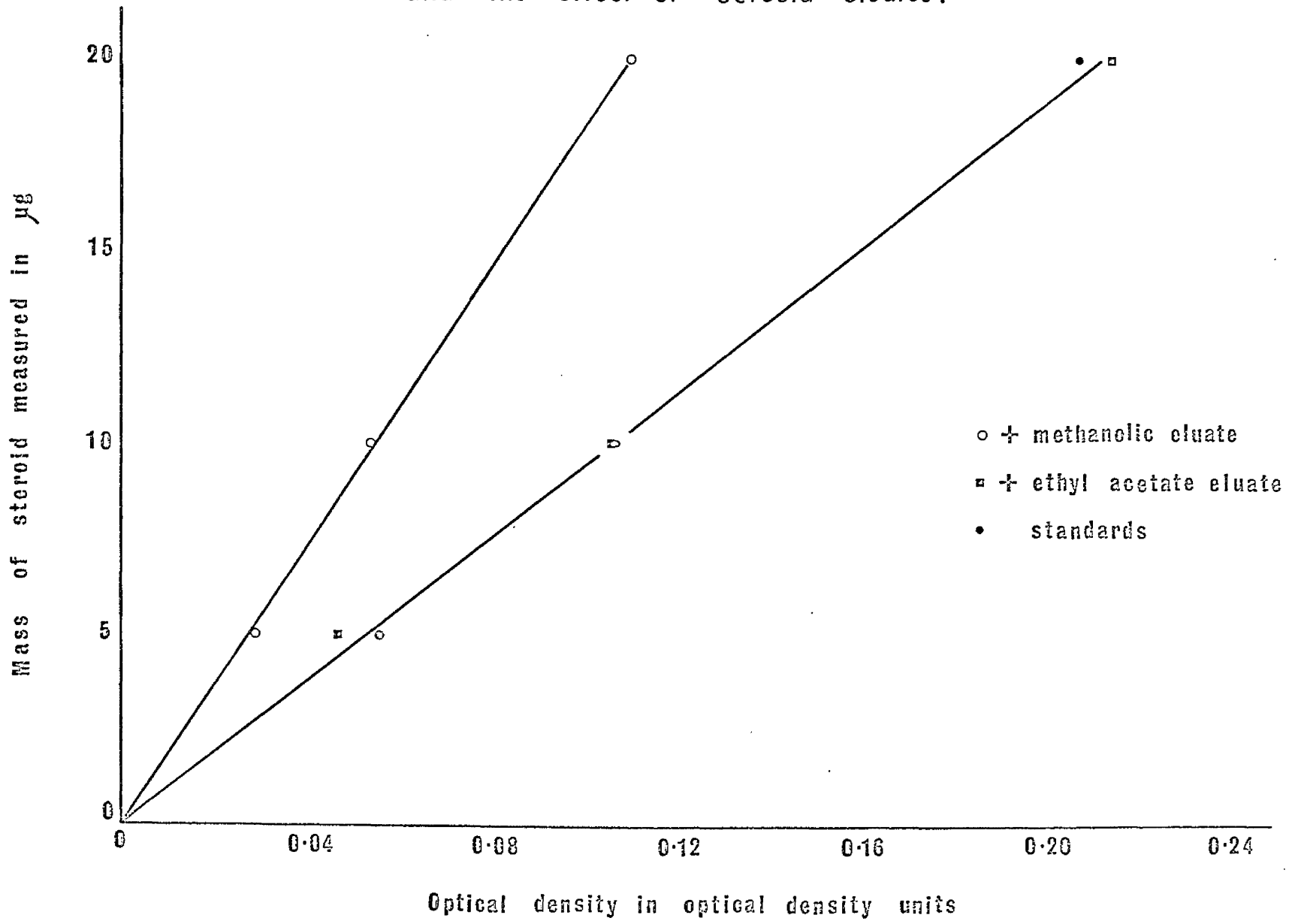
Four of these areas were subjected to the methanolic elution procedure and another four to that of ethyl acetate/saturated NaCl solution. Three eluates from each group were transferred to tubes containing, respectively, 5, 10 and 20 μg. DHA sulphate and the remaining two eluates were retained as blanks. A further set of tubes containing, again, 5, 10 and 20 μg. DHA sulphate were prepared as standard solutions for comparison.

Quantitation was achieved using the method of Crepy & Rulleau-Meslin (1960) (see Section IV, I iv). The results are shown below and the graphic representation of the Allen corrected optical density readings against mass is given in Graph 4.

Mass of steroid	Blank corrected optical densities (optical density units)		
	Standards	+ Methanolic eluate	+ Ethyl acetate eluate
5 μg.	0.056	0.029	0.047
10 μg.	0.107	0.054	0.106
20 μg.	0.207	0.110	0.214

From the results above and Graph 4 it is concluded

Graph 4. Quantitation of DHA sulphate using methylene blue and the effect of ecteola eluates.



that interference by ecteola/urea after elution of DHA sulphate with ethyl acetate/saturated NaCl solution is negligible whereas after using the methanol procedure a correction factor of 1.93 is required.

Appendix 6.

To assess the efficiency of elution of testosterone from a silica gel-coated plate using ethyl acetate.

Three aliquots of 10 µg. testosterone in ethanol were applied to a silica gel-coated plate. Without chromatography the three areas were scraped from the plate and the steroid eluted using ethyl acetate and water as described in Section IV, G. A further three similar areas of silica gel were also subjected to this elution procedure to provide blanks. Standard solutions, 3 x 10 µg., were prepared for comparative purposes.

The dried steroids and blanks were taken up in 5 ml. ethanol and the ultra-violet absorbancy determined using 1 cm. silica cells of an S.P.500 Spectrophotometer with light at 240 mµ.

Results.

(as optical density units).

		Optical density		Corrected for blank
Eluted steroid	1.	0.143		0.110
	2.	0.142		0.109
	3.	0.147		0.114
Blanks	1.	0.038		
	2.	0.030	0.033	
	3.	0.032		
Standards	1.	0.111		
	2.	0.112		
	3.	0.112		

The recovery of testosterone from silica gel by eluting with ethyl acetate from water is virtually 100%.

Appendix 7.

To assess the efficiency of elution of DHA sulphate from ecteola with ethyl acetate and saturated NaCl solution.

The investigations of metabolism and synthesis of steroid sulphates by testicular tissues necessitated the chromatography of these compounds on ecteola in the solvent system 4M.Urea in $3N.NH_4OH$ (see Section IV, K). It was therefore important to be able to elute these steroids from this material efficiently.

A stock solution A was prepared:-

7α-^{3}H DHA sulphate	-	0.5 μC.
Unlabelled DHA sulphate	-	100 μg.
Ethanol	-	10 ml.

Procedure.

(1) Preparation of standard solutions.

Three aliquots of 0.5 ml. of solution A were pipetted, individually, into vials and blown to dryness. Methanol (1.0 ml.) was added to each followed by 9 ml. scintillator solution.

(2) Preparation of standard solutions plus ecteola eluates.

A plate coated in ecteola was allowed to stand in a tank containing the solvent system 4M.Urea in 3N.NH_4OH until the solvent had run several cm. The plate was removed and dried. Three areas of 3 sq. cm. of the ecteola that had been traversed by the solvent system were scraped off the plate and extracted with 2 x 3 ml. ethyl acetate after the addition of saturated NaCl solution as in the procedure for eluting steroid sulphates (see Section IV, G). The eluates were dried and taken up in 2 ml. methanol. Three vials were taken containing in each case, 0.5 ml. solution A (evaporated to dryness), to which was added 1 ml. of the methanolic eluate of the three extractions, respectively, described above. Scintillator solution (9 ml.) was added to each vial.

(3) Elution of DHA sulphate from ecteola.

Three tubes containing 1 ml. solution A were taken and the solvent evaporated. To each were

added 3.0 sq. cm. of ecteola material from the pre-run plate. The steroids were extracted with 2 x 3 ml. ethyl acetate from saturated NaCl solution and blown to dryness. Each fraction was then dissolved in 2 ml. methanol and 1 ml. of each removed to a vial with 9 ml. scintillator solution.

(4) Preparation of blanks.

Three areas of pre-run ecteola (3 sq. cm.) were scraped from the plate and subjected to the ethyl acetate elution procedure. The dried eluates were dissolved in 2 ml. methanol and 1 ml. of each removed to a vial containing 9 ml. scintillator solution.

All vials were then placed in a Packard scintillation counter and radioactive content assessed.

Results.

(1)	Standard solutions	=	13,091 cpm.
(2)	Standards + ecteola eluates	=	13,490 cpm.
(3)	Extracted DHA sulphate	=	12,691 cpm.
(4)	Ecteola blanks	=	36 cpm.
(5)	Scintillator blank	=	40 cpm.

It can be seen from the results above that ecteola eluate does not suppress counting efficiency, in fact the counts from these fractions were slightly (2 - 3%) higher than the unaffected standards. The extracted DHA sulphate accounted for 97% of the initial radioactivity indicating a very good recovery by this technique, i.e. addition of saturated NaCl solution to the ecteola DHA sulphate complex and eluting with 2 x 3 ml. ethyl acetate.

Appendix 8.

To assess the efficiency of saponification of testosterone acetate and the recovery of testosterone after thin-layer chromatography.

To three tubes, each containing 20 µg. testosterone acetate in 1 ml. methanol, were added 0.25 ml. of a 2% solution aqueous potassium carbonate. The mixtures were allowed to stand overnight at room temperature.

The steroids were extracted by 3 x 3 ml. ethyl acetate after the addition of 3 ml. water to each tube, and chromatographed on silica gel in solvent system A (Table 4).

The ultra-violet absorbing spots corresponding to testosterone were eluted with ethyl acetate and recovery estimated by optical density determinations in ethanol.

Results.

	Optical density of testosterone	Silica gel blank	Corrected reading	% recovery
From tube 1.	218	35	183	91.0%
From tube 2.	196	35	161	80.2%
From tube 3.	171	35	136	67.3%

The results above indicate that this method of saponification and subsequent extraction, chromatography and elution provides a recovery of between 70 and 90% of testosterone from testosterone acetate.

Appendix 9.

To investigate the counting efficiency of 7α-^{3}H DHA sulphate after "quenching" with methanol and also the degree of "glass-sticking" with the lower levels of steroids used in this investigation.

The steroid sulphates are very polar compounds providing difficulties during the measurement of radioactivity with the toluene-scintillator solution used in these investigations. The problem may be overcome by dissolving the conjugates in 1 ml. methanol in a vial prior to the addition of 9 ml. scintillator solution. This technique affords solubility of the steroid but also results in attenuation of the pulse heights (quenching) during the measurement of radioactivity. This effect may be corrected for by employing an external quenched standard.

"Glass-sticking" is the term applied to the phenomenon of very small amounts of steroid that are said to adhere to glass surfaces. The use of larger amounts of steroid (e.g. 10 μg.) is thought to reduce this effect to a negligible level.

These problems were investigated as follows:

A known and equal amount of 7α-^{3}H DHA sulphate was used for each measurement (approx. 0.2 μg.).

(1) 7α-^{3}H DHA sulphate was dried in a vial and 10 ml. scintillator solutions added.

(2) 7α-^{3}H DHA sulphate was dried in a vial + 1 ml. methanol + 9 ml. scintillator solution.

(3) 7α-^{3}H DHA sulphate, pre-dissolved in 0.5 ml. methanol, was transferred to a vial and a further 0.5 ml. methanol and 9 ml. scintillator solution added.

(4) 7α-^{3}H DHA sulphate was placed in a vial and 10 μg. of the unlabelled steroid added in 1 ml. methanol followed by 9 ml. scintillator solution.

(5) 7α-^{3}H DHA sulphate, pre-dissolved in 1.0 ml. methanol, was transferred to a vial and a further 1.0 ml. methanol and 8 ml. scintillator solution added.

(6) Carbon-labelled and tritiated toluene were measured accurately into 3 vials each. To one of each was added 10 ml. scintillator solution

and to the remainder, 1 ml. or 2 ml. methanol and the volumes made up to 10 ml. with the requisite amount of scintillator. These vials served as normal and variably-quenched standards.

Results.

Effect of methanol on the efficiencies of counting ^{3}H and ^{14}C toluene.

(a) Unquenched ^{14}C-labelled toluene = 50.87%
^{14}C-toluene + 1 ml. methanol = 30.54%
^{14}C-toluene + 2 ml. methanol = 20.88%

(b) Unquenched ^{3}H-labelled toluene = 18.26%
^{3}H-toluene + 1 ml. methanol = 15.13%
^{3}H-toluene + 2 ml. methanol = 12.57%

It may be seen from the results given above that the addition of 1 ml. or 2 ml. methanol reduces the counting efficiency of the ^{14}C-toluene by 20% and 30% respectively. The efficiency of counting of the ^{3}H-toluene was similarly reduced by 3 and 6% respectively.

Results using 7α-^{3}H DHA sulphate.

Radioactivity (dpm) as determined by comparison with the appropriate preparation of radioactive toluene.

(1) 939,180

(2) 1,861,820

(3) 1,864,980

(4) 1,913,630

(5) 1,959,080.

These data show the increased counting efficiency of 7α-^{3}H DHA sulphate by liquid scintillation spectrometry of approximately 100% by the addition of 1 or 2 ml. methanol to the samples. The addition of unlabelled steroid (vial 4) shows little effect on the measurement of the contained radioactivity and indicates that "glass-sticking" is not of importance at the 0.2 μg. level.

Appendix 10.

Investigation of the loss of radioactivity when 7α-^{3}H androst-5-ene-diol is subjected to an oxidation reaction.

A solution containing 50 μg. unlabelled DHA and approximately 0.3 μC 7α-^{3}H DHA was prepared and chromatographed on a silica gel-coated plate in solvent system A (Table 4). The steroid was eluted and a specific activity determined. The remainder was subjected to a reduction reaction (see Section IV, H iv) and the resulting androst--5-ene-diol chromatographed on silica gel, again in system A. This provided the 7α-^{3}H androst--5-ene-diol to be investigated. The specific activity of this compound was determined and the remainder subjected to an oxidation reaction (see Section IV, H i). The products of this procedure were chromatographed on silica gel in system A and 6-oxo-androstenedione eluted. Its specific activity was assessed.

Results.

Steroid	Specific activity (dpm/mμmole)
DHA	25,649
androst-5-ene-diol	23,877
6-oxo-androstenedione	2,848

It may be seen from the results given above that oxidation using Kiliani's reagent causes loss of 7α-tritium from androst-5-ene-diol. It is assumed that this is due to enolisation at the 6,7 position. This procedure was therefore only used in the examination of carbon-labelled androst--5-ene-diol.

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